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Developmental Potential Of Rat Myoblast Lineages

Christopher Louis Pin

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Developmental Potential of Rat Myoblast Lineages

by

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of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Skeletal muscle consist of multinucleated fibres which are classified as slow (I) or fast (IIA, IIB, IIX) muscle fibre types which differ in their contractile properties and their expression of contractile protein isoforms. Individual muscles contain characteristic distributions of fibre types which can be identified based on their myosin heavy chain (MyHC) content. While classical studies have demonstrated that the fibre type composition of adult muscles can be altered by extrinsic factors (such as changes in the pattern of innervation), recent studies suggest that different fibre types may be formed from distinct myoblast lineages. The purpose of this study was to test the hypothesis that myoblasts from early and late stages of development represent distinct myoblast lineages, which differ in their developmental potential. The goals of this project were to (a) determine if developmental populations of myoblasts in the rat exhibit different fibre type potentials, (b) determine when lineages are established during myogenesis, and (c) determine if extrinsic factors can modulate their expression.

To address these questions, myoblasts obtained from embryonic day (ED) 14 (embryonic) and ED20 (fetal) rat hindlimbs were grown in culture and analyzed for MyHC expression using a panel of monoclonal antibodies specific for MyHC isoforms. Embryonic myoblasts expressed both embryonic and slow MyHCs while fetal myoblasts expressed embryonic, neonatal fast and adult fast isoforms, suggesting that these populations have different default patterns of expression and may be programmed to form slow or fast fibre types, respectively. To determine if these two populations could fuse with each other and if fusion altered MyHC expression, individual populations were specifically labelled and then co-cultured. Examination of these co-cultures revealed muscle heterokaryons containing nuclei from both embryonic and fetal myoblast populations. Individual nuclei maintained their characteristic MyHC expression as nuclear domains within these muscle heterokaryons indicating that distinct developmental potentials are established prior to fusion.

To determine if external cues could affect the developmental potential of these myoblast populations, both embryonic and fetal myoblasts were injected in the caudate-putamen of adult rats. Myotubes expressing slow, IIA, IIB and IIX MyHCs were observed in both types of injection grafts. However, myotubes which expressed exclusively slow MyHC were only found in the embryonic injection sites, indicating the existence of a "slow only" myoblast population only at early time points in development. Therefore, it appears that fetal myogenic precursor cells are present in embryonic cultures, but only differentiate *in vivo*. This suggests that the establishment of myogenic lineages is an early event in myogenesis, and may precede the expression of myogenic regulatory factors. In addition, the appearance of all adult MyHC isoforms preceded the disappearance of NCAM indicating that the initial production of MyHCs is not dependent upon innervation.

To further examine the role of extrinsic factors on muscle development, the developmental potential of L6 rat myoblasts was examined *in vitro* and following injection into regenerating adult muscles. Upon differentiation *in vitro*, L6 myotubes expressed predominantly embryonic and adult fast IIX MyHCs as well as a small amount of embryonic slow MyHC. Upon injection into regenerating muscles, L6BAG-A4 myoblasts expressing a Lac-Z reporter gene formed homotypic (donor only) and heterotypic (host/donor) fibres. Analysis of homotypic fibres revealed that fibres, which became innervated, exhibited embryonic and IIX MyHCs typical of the L6 *in vitro* phenotype. Analysis of heterotypic fibres in which the host contribution was typically fast IIA or IIB revealed the maintenance of IIX MyHC expression. However, analysis of L6 cells fusing onto slow fibres, revealed a switch in the phenotype of L6- derived nuclei to a slow phenotype, indicating repression of the IIX MyHC expression observed *in vitro*. Therefore, the L6 cell line appears to exhibit a fast IIX myoblast lineage that can be modulated by the environment.

These studies support the existence of myoblast lineages and suggest that, although environmental cues can modulate fibre type expression, the intrinsic program of the myogenic lineage determines the extent of modulation that can occur.

This restriction in developmental potential represents the adaptive range of a myoblast lineage. The "delineation" of muscle precursor cells with different developmental potentials follows myogenic determination and precedes myogenic differentiation.

To my parents, Ugo and Carol, who have always been there for me. What I am today is a result of what you taught me yesterday. Thank you.

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LIST OF ABBREVIATIONS

1° - primary

2° - secondary

A - adenine

ABC-AP - avidin biotin complex conjugated to alkaline phosphatase

ATP - adenosine triphosphate

BAG - β-galactosidase containing

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

BRDU - 5' - bromodeoxyuridine

BSA - bovine serum albumin

°C - degrees Celsius

Ca^{2+} - calcium

$CaCl_2$ - calcium chloride

CMF-HBSS - calcium and magnesium free Hank's Balanced Salt Solution

CO_2 - carbon dioxide

DAB - diamino benzidine

ddH_2O - double distilled water

D-MEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

ED - embryonic day

FITC - fluorescein isothiocyanate

g - gram

GAM IgG - goat anti-mouse immunoglobulin G

GAR IgG - goat anti-rabbit immunoglobulin G

HAM IgG - horse anti-mouse immunoglobulin G

HCl - hydrochloric acid

H_2O_2 - hydrogen peroxide

hr - hour

[^{3}H]Tdr - tritiated thymidine

HRP - horse radish peroxidase

kg - kilogram

lacZ - β-galactosidase gene

LTR - long terminal repeat

Mab - monoclonal antibody

MEF - myocyte enhancing factor

Mg^{2+} - magnesium

$MgCl_2$ - magnesium chloride

mg - milligram

min - minute

ml - millilitre

mM - milli Molar

mm - millimeter

mrf - myogenic regulatory factor

mRNA - messenger ribonucleic acid

N - normal

NaCl - sodium chloride

NaOH - sodium hydroxide

NBT - nitro blue tetrazolium

NCAM - neural cell adhesion molecule

neo^R - neomycin resistant

NFDM - non-fat dry milk

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PO_4 - phosphate

RAM-IgG_{2A} - rabbit anti-mouse immunoglobulin G subclass 2A

RT - room temperature

S phase - DNA synthesis phase

SDS - sodium dodecyl sulphate

SV 40 - simian virus 40

T - thymidine

TC - tissue culture

Tn5neo - neomycin resistance gene

TRITC - tetramethyl rhodamine isothiocyanate

V - volts

Vhrs - voltage hours

vol - volume

W - watts

X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactoside

% - percentage

α-MEM - alpha - Modified Eagle Medium

β-gal - β-galactosidase enzyme

μCi - microcurries

μg - microgram

μm - micrometer

CHAPTER 1 - HISTORICAL REVIEW

1.1 Structure of Skeletal Muscle

Adult mammalian muscle consists of long, multinucleated fibres which extend the entire length of the muscle bed. Individual fibres can be classified based on their speed of contraction (Buller *et al.* 1960), resistance to fatigue (Gauthier, 1986) and expression of different muscle-specific proteins (Pette and Staron, 1990). Although early studies suggested the existence of two general classes of muscle fibres, fast and slow, more recent evidence indicates that the fast class is further subdivided into three sub-classes; IIA, IIX and IIB (LaFromboise *et al.* 1990), which also exhibit differences in their contractile abilities (Schiaffino *et al.* 1989). Each muscle contains different proportions of fibre classes, presenting a characteristic pattern of fibre-typing (Armstrong and Phelps, 1984). Muscle fibre populations exhibit differences in their type of innervation with fast and slow fibres being innervated by fast and slow classes of motoneurons, respectively (Burke, 1986). Currently, no differences have been observed within the motoneuron population innervating the three sub-classes of fast fibres. However, fibres can be grouped together into motor units, which consist of similar muscle fibres innervated by a single motoneuron (Grinnell, 1986). In spite of this, the mechanisms which account for differences in the pattern of muscle fibre types between individual muscles are unknown.

1.2 Hindlimb Muscle Development

During normal myogenesis, there are three major events involved in the formation of mature muscle. The first step, myogenic determination, involves the development of pleuripotential mesodermal cells into myogenic stem cells, which are determined to form muscle but have not yet activated the myogenic regulatory factor genes (Tajbakhsh and Buckingham, 1994). The second event is myogenic differentiation, which begins with the transition of a muscle precursor cell into a myoblast concomitant with the expression of myogenic regulatory factors, such as

myf-5. These myoblasts eventually fuse to form multinucleated myotubes, which express a battery of muscle - specific contractile protein genes (Konieczny and Emerson, 1985). Finally, myotubes mature into muscle fibres which have peripherally located nuclei, well developed sarcomeres and express adult MyHC isoforms.

At present, it is uncertain where along the myogenic pathway these cells become committed to a specific fibre type. However, it has been suggested that myogenic lineages are established prior to innervation (Stockdale, 1990; Hoh *et al.* 1989). The term "lineage" has been used previously by developmental biologists in reference to a population or populations of cells which share a common point of origin. In other words, cells which are derived from a common precursor stem cell are said to belong to the same lineage. However, the strict application of this definition would imply that all cells belong to the same lineage since they are all originally derived from the zygote. Therefore, it is necessary to restrict the definition of the word, lineage. In this thesis, the term "myogenic lineage" refers to a population of myoblasts which share a common phenotypic fate, in which this fate is established prior to fusion as suggested by Donoghue and Sanes (1994). In the strictest sense, myoblast lineages would develop according to intrinsic programs which would restrict the phenotypic potential of the cell, overriding any environmental stimuli that are present. Although several classical studies involving the cross-innervation of different muscles suggested that muscle phenotype is governed strictly by the type of innervation present (Pette and Vrbova, 1985; Buller *et al.* 1960; Jolesz and Sreter, 1981; Carraro *et al.* 1986), recent work indicates that under some experimental conditions, muscle fibres may be refractory to environmental influences (Thomas and Ranatunga, 1993; Graybiel, 1990). The appearance of a population of muscle fibres that is not affected by changes in innervation (Thomas and Ranatunga, 1993) has led some researchers to suggest that intrinsic differences between different myoblast lineages may significantly contribute to the development of muscle fibre type (Stockdale, 1992; Stockdale, 1990).

1.2.1 Development of Rat Hindlimb Muscle

The hindlimb musculature is the result of two waves of myoblast fusion. Around ED 14, myoblasts accumulate in two small pouches in the mid - caudal region of the fetus, known as the hindlimb buds (Condon *et al.* 1990). These myoblasts align into long rows which stretch from tendon to tendon, and then fuse with each other (Duxson and Usson, 1989). By ED 17, the final number of these primary (1°) myotubes is reached (Ross *et al.* 1987). It is also at this time that secondary (2°) myotube formation starts. Using the 1° myotubes as a scaffold, myoblasts start to fuse with each other and form 2° myotubes which will form the bulk of the muscle in the adult (Duxson *et al.* 1989). 2° myotubes are continually added until postnatal day 7 (PN 7), at which time they can outnumber the 1° myotubes by a nine to one ratio (Wilson *et al.* 1988).

The actual fusion kinetics for each myotube population are quite different. The myoblasts which form 1° myotubes initially align in an arrangement stretching the length of the muscle. These myoblasts, which have similar birth dates, then fuse in a synchronous manner and insert immediately into the tendon (Ross *et al.* 1987). These insertions are already quite mature by the time the second wave of fusion occurs, and by ED 21 will be comparable to adult myotendonous junctions (Duxson and Usson, 1989). Once fused in this manner, the 1° myotubes will grow by synthesis of new cytoplasm only, and not by the further fusion of other myoblasts (Harris *et al.* 1989). These myotubes develop into a stable framework that allows the completion of muscle development. It is believed that the delay between 1° and 2° myogenesis allows the 1° myotubes to become strong enough to support further muscle growth (Ontell and Kozeka, 1984).

As already mentioned, a second population of myotubes appears in the hindlimb region around ED 17 (Ross *et al.* 1987). The muscle precursor cells for these myotubes can be found in the basal lamina of the 1° myotubes (Harris *et al.* 1989). The initial fusion process to produce 2° myotubes occurs within this basal lamina, directly over the motor endplate of the 1° myotube (Harris *et al.* 1989). At

this point, two myoblasts from the secondary myoblast population can fuse to start a 2° myotube. The 2° myotubes then grow through the fusion of myoblasts at random points along their length. The fusion of 2° myotubes is, therefore, an extended process lasting several days in which myotubes continue to grow until they reach the tendon on either end (Duxson *et al.* 1989). Although myoblasts which will form 2° myotubes are located within the basal lamina of 1° myotubes, it has been suggested that fusion does not take place between myoblasts of different populations (Duxson *et al.* 1989). This suggests that the fusion process is very selective, with 2° myoblasts fusing only to 2° myotubes, and not to 1° myotubes. These observations suggest that the myoblasts which form 1° myotubes are different and may represent different myogenic lineages.

1.2.2 Control of 2° Myotube Formation

Since 2° myotubes usually originate near motor endplates (Duxson and Usson, 1989), it has been suggested that their formation is regulated in some way by the nerve (Pette and Vrbova, 1985; Wilson and Harris, 1993). This hypothesis is supported by the observation that following partial denervation of rat fetal muscles prior to 2° myotube formation, the number of 2° myotubes decreases proportionately with the amount of denervation (Ross *et al.* 1987). This same procedure does not seem to effect formation of 1° myotubes. Several mechanisms have been proposed to explain this relationship. Sheard et al (1991) proposed that 2° myotubes are activated by end - plate potentials that originate in the adjacent 1° myotube and are transported via gap junctions. In contrast, Duxson et al (1989) have suggested that a signal from the nerve terminal induces a mononucleated cell to be receptive to fusion.

However, recent results with aneural muscles have further complicated the story by showing the development of 2° myotubes occurs normally in aneural muscles (Fredette and Landmesser, 1991; Condon *et al.* 1990). Condon et al (1990) argue that previous experiments in which muscles were totally denervated caused 1°

myotube degeneration, thereby affecting the structural framework necessary for proper development. The denervation of the hindlimb caused extensive distortion of the individual muscles, while some muscles were even lost. Since Condon's results demonstrate that 2° myogenesis can still occur in the absence of innervation, the role of innervation in myoblast fusion remains controversial.

1.2.3 Involvement of Neural Cell Adhesion Molecules (NCAMs) in Myogenesis

Neural cell adhesion molecules (NCAMs) belong to the super family of IgG proteins (Cunningham *et al.* 1987) and are specifically localized to the membrane of neurons and cell types that may interact with these neurons (Linnemann and Bock, 1989). These proteins are thought to facilitate cell adhesion through homophilic binding of NCAMs on adjacent cell surfaces (Rutishauser *et al.* 1995). Several different isoforms of NCAM exist, and these are the products of alternative splicing of a single copy gene (Thompson *et al.* 1989; Goridis and Wille, 1988). In muscle, three such isoforms are expressed (Moore *et al.* 1987; Lyons *et al.* 1992), one of which contains a muscle-specific domain (msd) (Dickson *et al.* 1987). At early time points in mouse gestation, the 180 and 140 kD NCAM isoforms can be observed within the myotome (Moore *et al.* 1987). The NCAM isoform containing the msd is not expressed until ED 11 in the mouse. As the levels of the latter isoform increase, levels of the 180 and 140 kD isoforms decrease. However, starting at ED 13, levels of all isoforms start to decline (Moore *et al.* 1987). The expression of NCAM within muscle undergoes a transition with a putative embryonic form at high levels early in development and a more mature isoform existing later in development (Rieger *et al.* 1985; Covault and Sanes, 1986). The embryonic isoform shows a heavy concentration of polysialic acid, which is thought to modulate cell adhesion (Rutishauser and Landmesser, 1991), while the mature isoform has a much lower level of polysialylation. The transition from one isoform to the other coincides with myoblast fusion (Covault *et al.* 1986; Moore *et al.* 1987), and results in altered binding properties of the NCAMs (Hoffman and Edelman, 1983). Therefore, it has

been suggested that the embryonic form may be functionally linked to myoblast fusion. However, since antibodies specific for NCAM do not affect the fusion of myoblast populations, it has been postulated that NCAM may only mediate the calcium-independent aspect of myoblast adhesion (Mege *et al.* 1992; Rutishauser *et al.* 1988).

The mature form of NCAM has been implicated in the establishment and maintenance of a neuromuscular junction on mature muscle fibres (Landmesser *et al.* 1988) as well as in the separation of 2° myotubes from the supporting 1° myotube during normal muscle development; a process known as fasciculation (Fredette and Landmesser, 1991). Interactions between nerve and myoblasts can be blocked *in vitro* by the addition of F(ab) fragments of antibodies specific for NCAM (Grumet *et al.* 1982). Similar studies in which NCAM specific antibodies were introduced into denervated muscles disrupted the recovery of the neuromuscular junction (Remsen *et al.* 1990). Initially, NCAM is uniformly distributed along the entire surface of myotubes (Covault and Sanes, 1986). Upon innervation this protein is undetectable in portions of the fibres distant from the synapse (Rieger *et al.* 1985) and becomes specifically localized to the sub- and perisynaptic regions. Antibodies specific for NCAM will only recognize muscle fibres at the time of synapse formation and therefore, can be used to follow fibres' state of innervation (Moore and Walsh, 1985). When muscle is denervated, the level of NCAM expression increases to the levels exhibited in development (Moore and Walsh, 1986; Muller-Felber *et al.* 1993). Similar increases were observed in regenerating muscle (Irintchev *et al.* 1994), suggesting that a lack of innervation is causing the up-regulation of NCAM. In fact, the up-regulation of the NCAM isoform containing the msd is believed to make the muscle more susceptible to innervation (Moore and Walsh, 1986). Subsequent re-innervation of the damaged muscle results in the immediate down-regulation of this NCAM to adult levels, and are specifically localized to the motor endplate region (Covault and Sanes, 1985). Also, the formation of neuromuscular junctions occurs around postnatal day 14 (Ontell and Kozeka, 1984) which

corresponds to the down-regulation of NCAM (Lyons *et al.* 1992). This expressional pattern mimics the expressional pattern of acetylcholine receptors, which become specifically localized to the synaptic region upon innervation (reviewed by Fambrough, 1979). Therefore, the localization of NCAM specifically to the motor endplate and the loss throughout the remainder of the sarcolemma, indicate that innervation has occurred.

1.3 Myogenic Regulatory Factors (mrfs)

Within the last eight years, several genes belonging to the helix-loop-helix family of proteins have been discovered that play pivotal role in muscle differentiation (reviewed by Olson, 1990; Rudnicki and Jaenisch, 1995). This group, known as the myogenic regulatory factors (mrfs), includes MyoD (Davis *et al.* 1987), myogenin (Wright *et al.* 1989), myf-5 (Braun *et al.* 1989), and MRF4 (Rhodes and Konieczny, 1989). MyoD was the first mrf to be discovered, and transfection of the MyoD gene into C3H10T1/2 cells caused transformation of this fibroblast cell line into muscle cells and fibres (Davis *et al.* 1987). Several other genes were subsequently isolated, all showing the ability to induce myogenesis in fibroblast cell lines, as well as adipocyte and chondrogenic cell lines (Wright *et al.* 1989; Davis *et al.* 1987). These genes have now been shown to exist in quail (de la Brousse and Emerson, 1990), mice (Davis *et al.* 1987; Edmondson and Olson, 1989; Miner and Wold, 1990), rats (Rhodes and Konieczny, 1989; Wright *et al.* 1989), *Caenorhabditis elegans* (Krause, 1995), drosophila (Abmayr *et al.* 1992) and humans (Braun *et al.* 1989; Braun *et al.* 1990), with cDNA probes being available for all of them.

Mrf proteins localize to the nucleus, indicating direct interactions with muscle - specific genes (Brennan and Olson, 1990). These proteins have been shown to have a DNA - binding region, which is specific for the sequence CANNTG (Olson, 1990; Weintraub *et al.* 1991), otherwise known as the E box, found in promoter regions of many structural muscle genes. This E box has also been found in

promoter regions of non muscle genes, indicating that the helix-loop-helix motif may be common to other regulatory genes (Buskin and Hauschka, 1989). The binding of these mrf proteins seems to occur in heterodimer fashion, with concurrent binding of ubiquitous proteins, such as E12 or E47 (Olson, 1990). These mrf genes appear in skeletal muscle cells and fibres exclusively, and transfection of one mrf will lead to the transcription of other mrfs (Rhodes and Konieczny, 1989). This has led researchers to suggest a model in which these genes interact in some form of cascade.

1.3.1 Developmental Expression of Myogenic Regulatory Factors

In determining the functional differences between the different myogenic regulatory factors, one of the first steps taken was to characterize these genes with respect to their initial expression and absolute levels during the developmental period. Mrf expression is first detected in early development for both mice and rats (ED8 and ED10, respectively) (Hannon *et al.* 1992; Smith *et al.* 1993; Hinterberger *et al.* 1991). A highly specific pattern has been shown for each of the four myogenic regulatory factors, with somitic and hindlimb muscles showing differential expression. In mouse somites, the first mrf turned on is myf-5 (Ott *et al.* 1991). This expression starts at ED 8 in the mouse (Sassoon *et al.* 1989) and is followed by myogenin, approximately one day later, at ED 9.5. This expression is seen at the mRNA level, with recent evidence showing that the protein is not present until ED 10.5 (Cusella-DeAngelis *et al.* 1992). MRF4 expression follows (Hinterberger *et al.* 1991), with MyoD being the last mrf to be expressed (mRNA detected at ED 10.5 (Sassoon *et al.* 1989). The expression of these mrfs seems to be in an anterior - posterior gradient within the somites, (Rhodes and Konieczny, 1989) with MyoD being the last mrf expressed at the most posterior aspect of the somites. This pattern of expression is similar to that reported in rat fetal development, with the onset of mrf expression occurring two days later due to the differences in gestational length (Rhodes and Konieczny, 1989).

This same pattern of expression is not found in hindlimb muscle development,

however. Myf-5 expression still proceeds the other mrfs, first being seen at ED 11.5, and this is followed quickly by myogenin (Ott *et al.* 1991). The differences lie in the expression of MRF4 and MyoD. MyoD expression appears concurrently with myogenin, while MRF4 does not appear until later in hindlimb development (ED 14) (Hinterberger *et al.* 1991). The expression of MyoD and myogenin correspond to the initial presence of 1° myotubes, while myf-5 appears before any fusion occurs (Ott *et al.* 1991). The expression of these three mrfs continues to rise until ED 14, at which time myf-5 expression decreases, eventually disappearing after birth (Ott *et al.* 1991). MyoD and myogenin continue to increase until birth, decreasing postnatally until they are found only at low levels within the adult. MRF4 expression, however, is much different, with expression beginning at ED 14 and increasing into adulthood, where it is maintained at high levels (Hinterberger *et al.* 1991). The initial expression of MRF4 corresponds to the appearance of 2° myotubes in development, and it has been suggested that this may represent a difference in the function of this mrf (Hinterberger *et al.* 1992; Montarras *et al.* 1991). The approximate down-regulation of these factors have also been established. The first to disappear is myf-5, which is not detectable after ED 14 in the mouse (Cusella-DeAngelis *et al.* 1992), but seems to linger longer in rats (Rhodes and Konieczny, 1989). The other three factors do not disappear in the adult. However, it has been shown that MyoD and myogenin are maintained in very low levels, while MRF4 is the only mrf to be maintained at high levels (Hinterberger *et al.* 1991). Because of this pattern of expression it has been suggested that myogenin and MyoD cause transition of myoblasts into myotubes while MRF4 is a maintenance regulatory protein in differentiated fibres (Montarras *et al.* 1991)

1.3.2 Mrf Knock-out Experiments

To elucidate the individual roles of mrf's, investigators have recently examined the phenotypes of mice in which a specific mrf is missing (Zhang *et al.* 1995; Rudnicki *et al.* 1992; Braun *et al.* 1992; Rudnicki *et al.* 1993; Braun and

Arnold, 1995; Nabeshima *et al.* 1993; Hasty *et al.* 1993). This involved the establishment of transgenic mouse lines in which each gene had been removed or "knocked-out". The first such knock-out experiments involved either myf-5 (Braun *et al.* 1992) or MyoD (Rudnicki *et al.* 1992), the first two mrf's to be expressed during early development. The individual knock-out of myf-5 resulted in homozygotes that died soon after birth (Braun *et al.* 1992). However, this was not due to an absence of muscle, but rather to the absence of a developing rib cage. In fact, although the appearance of myotubes was delayed slightly, the final phenotype of the muscle was normal. When the similar experiment was performed with MyoD, the results were even less dramatic, with no apparent phenotypic abnormality of the mice (Rudnicki *et al.* 1992). Observations that myf-5 expression increased in MyoD negative homozygotes suggested that these mrf's may be functionally redundant.

Double knock-out experiments involving myf-5 and MyoD resulted in mice in which no muscle was present (Rudnicki *et al.* 1993). There was a complete absence of both muscle and myoblasts indicating that myogenic determination will not take place in the absence of these two factors. Although other abnormalities existed within these homozygotes (such as a cleft palate), these were thought to be secondary to the absence of skeletal muscle. These experiments suggested that myf-5 and MyoD are involved in the early determination of myogenic precursor cells into myoblasts, and that they may have redundant functions at this stage (Braun *et al.* 1992).

The next mrf to be knocked-out was myogenin (Nabeshima *et al.* 1993; Hasty *et al.* 1993). This single knock-out experiment resulted in homozygous fetuses lacking skeletal muscle. Upon closer examination, mononucleated myoblasts were observed in all areas where skeletal muscle would have formed. This suggests that myogenin plays a specific role in the differentiation of myoblasts into myotubes, and acts downstream of both myf-5 and MyoD (Venuti *et al.* 1995). Interestingly, culturing of myogenin-negative myoblasts in culture has little effect on the myogenic pathway, with myotubes being formed. It has been suggested that this *in vitro* fusion

of myogenin-negative cells is due to the ability to limit the growth factors present in culture which prevent fusion *in vivo*.

The most recent knock-out experiment to be performed involved MRF4 (Zhang *et al.* 1995), which is believed to appear last in the myogenic cascade pathway of mrf's (reviewed by Edmonson and Olson, 1993; Sassoon, 1993). These experiments resulted in no significant alterations to muscle differentiation, although subtle differences in muscle protein expression were observed. However, along with the absence of MRF4, there was a concomitant increase of expression of myogenin, indicating that myogenin may be replacing MRF4 functionally. Once again, several rib abnormalities were observed, including extensive bifurcations and fusions (Zhang *et al.* 1995). Therefore, it seems that MRF4 has a role downstream of myogenin after fusion has occurred and that it may influence rib formation through indirect mechanisms, possibly in concert with myf-5.

These studies have confirmed the cascade pathway of mrf expression observed in hindlimb muscle development (reviewed by Sassoon, 1993). The first factor to appear is myf-5 followed shortly by MyoD. Myogenin then appears at slightly later time points and MRF4 appears last. This pattern of expression is consistent with the model that myf-5 and MyoD are involved in myogenic determination, while myogenin and MRF4 play roles in myogenic differentiation and maturation (Montarras *et al.* 1991). However, recent evidence indicates that myogenic determination is established prior to the expression of the mrf's (Tajbakhsh and Buckingham, 1994). This suggests that there may be other members of this family which are upstream in the cascade pathway. Recent observations have indicated that one such gene may be PAX-3 (Williams and Ordahl, 1994).

1.3.3 Mrf Expression in Various Cell Lines

To date, all myoblast cell lines established share a common feature in that they all express myf-5 in the period prior to differentiation and fusion (Montarras *et al.* 1991). Myf-5 positive/MyHC negative cells have been shown in both rat L6

(Hinterberger *et al.* 1991) and mouse C2C12, Sol8, and BC3H1 (Montarras *et al.* 1991) myoblast lines. All of these lines also express myogenin, which usually accompanies differentiation (Montarras *et al.* 1991). The expression of these mrf proteins is not transient since they are still found in fully differentiated myotubes (Hinterberger *et al.* 1991). MyoD expression, which has been suggested to initiate muscle differentiation, is not present in L6 (Hinterberger *et al.* 1991) or BC3H1 (Davis *et al.* 1987; Edmondson and Olson, 1989) myoblasts, while MRF4 is not found in BC3H1 cells (Block and Miller, 1992). The developmental expression of these last two mrfs *in vitro* is similar to that found *in vivo*. MyoD can appear before, or coincident with differentiation and myogenin expression, while MRF4 is usually found in more mature fibres (Rhodes and Konieczny, 1989; Hinterberger *et al.* 1991).

The fact that MyoD and MRF4 are not found in some myoblast lines implies that one mrf may compensate for the absence of another mrf, since normal myogenesis occurs in most cases. L6 cells show an abnormally high amount of myf-5 expression (Hinterberger *et al.* 1991), implying a possible backup mechanism built into the muscle regulatory pathway. However, transfection of various myoblast lines with mrfs has suggested that individual roles for the mrf's may exist. When MRF4 is transfected into BC3H1 cells, these myoblasts acquire the ability to fuse, and express fast myosin light chain 1 and MyoD, unlike the normal, non-transfected BC3H1 cell line (Block and Miller, 1992). Since MyoD is turned on, it is difficult to tie MRF4 expression to the other two functions. However, these differences were not observed upon transfection of the same cell line with MyoD (Block and Miller, 1992). Another transfection study (Muthuchamy *et al.* 1992) provides support for differential roles of the mrf proteins. L6 myoblasts transfected with MyoD showed expression of myosin light chain 1 and cardiac α-actin mRNA, which were previously not detected in normal L6 cells (see further discussion below).

Although MyoD appears to play a redundant role in myogenic determination, it has recently been suggested that it may have other functions such as the

establishment of fast fibre types. *In situ* hybridization has indicated that MyoD is expressed at relatively high levels in fast fibres (Hughes *et al.* 1993). Conversely, slow fibres express higher amounts of myogenin. Therefore, it has been postulated that these mrf's promote specific phenotypes within adult muscle. Although transformation of fast to slow muscle by cross-innervation is accompanied by changes in the reative levels of MyoD and myogenin (Voytik *et al.* 1994), there is no direct evidence that there is a causal relationship between the expression of the mrf's and the fibre types observed.

1.4 Myosin as a Determinant of Muscle Fibre Type

Myosin is one of a group of structural proteins whose main role is in the contraction of muscle cells. It also plays an integral part in cell motility in non - muscle cells, both eukaryotic and prokaryotic (Korn and Hammer, 1988). This protein is believed to represent up to 20% of the total protein in skeletal muscle cells (Nadal-Ginard, 1978). It is found in dark bands of sarcomeric arrangements within skeletal and cardiac muscle cells, combining with actin and tropomyosin to form a sarcomere, the major contractile unit. Native myosin is composed of six subunits - two alkali light chains, two regulatory light chains and two heavy chains. (reviewed in Emerson and Bernstein (1987). All of these subunits are produced by multi - gene families, in which different family members are expressed in a developmental and fibre type specific manner (Mahdavi *et al.* 1986; Whalen, 1985).

1.4.1 Gene Family and Fibre Typing

To date, nine different sarcomeric myosin heavy chain (MyHC) isoforms have been reported in rats (reviewed by Pette and Staron, 1990). These isoforms include two cardiac - type MyHCs (α and ß), one of which (β - cardiac) is expressed in slow skeletal muscle fibres (Mahdavi *et al.* 1982), three fast - type MyHCs (IIA, IIB, and IIX) (Schiaffino *et al.* 1989; LaFromboise *et al.* 1990), and two developmental MyHCs (embryonic and neonatal fast) (Lewis *et al.* 1982). The remaining two

MyHC isoforms are limited in their expression to one specific tissue: extraocular MyHC (Wieczorek *et al.* 1985) and superfast MyHC in the masseter muscle (Hoh and Hughes, 1991). Many of the isoforms have been characterized through cloning and sequencing in avians, rodents and humans (Periasamy *et al.* 1985; Jaenicke *et al.* 1990; Hoh *et al.* 1989; Molina *et al.* 1987; Stedman *et al.* 1988), and cDNA probes (Mahdavi *et al.* 1982; Medford *et al.* 1980) and monoclonal antibodies (Gambke and Rubinstein, 1984; Khaw *et al.* 1984; Bouvagnet *et al.* 1984) specific to many of the above isoforms have been developed. Recently, slow embryonic and slow neonatal isoforms have also been identified in the rat through immunohistochemistry (Hughes *et al.* 1993). However, neither of these isoforms have been shown to be transcribed from unique MyHC genes and no cDNA probes exist for either isoform.

At the protein level, most adult muscle fibres within an individual muscle express one predominant MyHC isoform (reviewed by Pette and Staron, 1990). Muscle fibre typing usually involves the discrimination of fibres based on their expression of MyHC isoforms. The classical histochemical reaction involves an ATPase reaction in which the pH of the preincubation wash will allow the different fibres to be recognized (Gauthier, 1986). Slow fibres remain stained for ATPase activity at a pH of 3.9, while IIA fibres show no reaction unless the pH is at least 4.7. IIB fibres lose their reaction at an intermediate pH, thereby allowing them to be differentiated from the IIA fibres (Brooke and Kaiser, 1970). It appears that the maximum velocity of shortening within a fibre is correlated to the MyHC isoform expressed, and that this velocity is related to the myosin ATPase activity (Reiser *et al.* 1985). Therefore, it seems that this ATPase fibre typing assay is directly related to the expression of MyHC isoforms within a fibre. There are some ambiguities, such as the presence of other MyHC isoforms (eg. IIX) which may appear to be the same as another fibre type under the same conditions (Schiaffino *et al.* 1989), or transitional fibres which express relatively high amounts of more than one type of MyHC isoform since they are in the process of changing their expression. These have been called IIC fibres (Brooke and Kaiser, 1970).

Although there are some instances in which more than one MyHC isoform can be expressed within a fibre, usually there is one form that predominates. Fibres that contain more than one MyHC type are usually in a state of transition, due to either environmental influences (Pette and Vrbova, 1992) or the normal pattern of development (Condon *et al.* 1990; Harris *et al.* 1989; Butler-Browne and Whalen, 1984). There is now evidence that the majority of muscles fibres contain trace amounts of other MyHC isoforms (Campione *et al.* 1993). A continuum of phenotypes is often observed with the co-expression of MyHCs restricted to isoforms with closely related contractile properties (Staron and Pette, 1993; DeNardi *et al.* 1993). For example, the slow MyHC was found to be co-expressed with fast IIA MyHC, but not fast IIX MyHC. However, due to the predominance of one MyHC isoform, it is still possible to classify muscle fibres based on their MyHC content. Therefore, type I, IIA, IIX, and IIB fibres contain predominantly slow, IIA, IIX and IIB MyHCs, respectively (Termin *et al.* 1989).

Different fibre types, based on their MyHC content, are very widespread (Armstrong and Phelps, 1984) and, with the exception of the tissue - specific MyHC isoforms such as the extraocular (Wieczorek *et al.* 1985), masseter (Hoh and Hughes, 1991) and α-cardiac (Lompre *et al.* 1984) isoforms, they can be found in almost all muscles within the rat. For instance, the β-cardiac isoform, which is the predominant MyHC type in fetal heart development (Mahdavi *et al.* 1982), is the same as slow - type MyHC which predominates in the soleus. Adult fast isoforms can be found in most skeletal muscles as well as in the diaphragm (Schiaffino *et al.* 1989) and extraocular (Wieczorek *et al.* 1985) muscles. The embryonic and neonatal fast isoforms are found almost exclusively during development with embryonic MyHC being the predominant isoform in all fibres during gestation (Whalen *et al.* 1981), and neonatal MyHC predominating at the time of birth (Weydert *et al.* 1987) After this time point, individual adult isoforms begin to predominate, and the developmental forms are only found within areas of regeneration (Whalen *et al.* 1990), and in the extraocular muscle (Wieczorek *et al.* 1985) of adults.

Individual muscles, such as the tibialis anterior and gastrocnemius, have a unique spatial arrangement of the slow and fast - type fibres (Armstrong and Phelps, 1984). One muscle will contain several different MyHC isoforms, and therefore, will be made up of several different fibre types. Within the hindlimb, the arrangement of fibre types is typically similar in a variety of muscles, with slow fibres being located deep to fast fibres, usually adjacent to the femur, tibia or fibula (Armstrong and Phelps, 1984). However, there may be some slow type fibres in predominantly fast areas, and vice versa. A major distinguishing feature between the different muscles in the hindlimb is that they contain different percentages of fast and slow type fibres. For instance, the soleus muscle in the rat is a predominantly slow muscle containing approximately 87% slow type fibres. This is an unusual occurrence since the hindlimb muscles in total contain only 5% slow fibres. The extensor digitorum longus in the rat, on the other hand, contains 99% fast type fibres. However, most muscles in the hindlimb will contain proportions of slow and fast fibres between these two extremes (Armstrong and Phelps, 1984). For example, the gastrocnemius is divided into two heads - the white, lateral head is typically fast, with few slow fibres, while the red, medial head contains a much higher percentage (30%) of slow fibres (Armstrong and Phelps, 1984). To determine how these localized areas of muscle fibre types have arisen, several developmental studies have been done to examine their initial establishment.

1.4.2 Developmental Expression of MyHC Isoforms in Rat Hindlimbs

As described earlier, muscle development in the rat hindlimb involves two periods of muscle fibre formation in which 1° and 2° myotubes develop (Ross *et al.* 1987). These myotubes can be distinguished by the gestational age in which they first appear (see Section 1.2.2) and by their initial myosin expression. 1° myotubes are first detected in the crural muscle of the hindlimb starting at ED 15, and then in the lumbrical muscles by ED 16 (Condon *et al.* 1990). Initially, these myotubes uniformly express only embryonic myosin with a subset expressing slow myosin

(Condon *et al.* 1990). It had previously been suggested that the slow MyHC expression does not occur until neonatal day 14 (Whalen *et al.* 1981), but by ED 17 the majority of 1° fibres co-express both embryonic and slow myosin (Condon *et al.* 1990). Starting at ED 17, a small subset of the 1° fibres also express neonatal myosin. Although the slow and neonatal isoforms are initially co-expressed in the 1° myotubes, they will eventually form complimentary populations (~ ED 20) with fibres expressing only one of the two isoforms (Harris *et al.* 1989). Transition of 1° myotubes from slow to neonatal occurs in a specific, spatial pattern, starting at the periphery of the muscles, and gradually spreading inward, until the slow fibres are localized to the deep part of the muscle, adjacent to the bones (Condon *et al.* 1990). The 1° fibre transition has been described in two different ways. Harris et al. (1989) suggest that all 1° fibres express neonatal and slow at the same time, and that selective innervation will determine which isoform will be turned off. In contrast, Condon et al. (1990) suggest that only the fibres that will eventually form the fast type fibres in the adult will go through the slow to neonatal transition, and that this process is not controlled by innervation. Further experiments have shown that both slow and neonatal populations of 1° fibres develop without innervation, suggesting that the latter hypothesis may be the correct model (Condon *et al.* 1990).

The second period of myoblast fusion starts around ED 18, depending on the location of the muscle within the hindlimb (Condon *et al.* 1990) and results in the formation of 2° myotubes. By ED 19, 2° myotubes are found in every muscle mass within the hindlimb (Ross *et al.* 1987). These fibres do not express the slow MyHC isoform, but uniformly co-express embryonic and neonatal MyHCs (Condon *et al.* 1990; Harris *et al.* 1989). The majority of these myotubes will go on to form fast fibres in the adult, with a few exceptions changing to slow fibres (usually in the soleus) (Narusawa *et al.* 1987). Other switches to slow fibres may also take place in the deep part of the muscles. (Harris *et al.* 1989) The fibre transition continues for at least four weeks after birth, and during this time, up to three different MyHC isoforms may be co-expressed (Kugelberg, 1976; Butler-Browne and Whalen, 1984).

1.5 Muscle Degeneration and Regeneration Following Damage Without Nerve Involvement

Muscle regeneration is an important process for skeletal muscle maintenance, and involves several specific events which are easily recognizable. The regenerative process is influenced by many different variables, the most important of which is the state of the basal lamina after the degeneration of the original muscle mass (Bischoff, 1974; Carlson, 1973). Muscle degeneration can be the result of several different types of trauma including toxins (Alameddine *et al.* 1989), cold (Stewart *et al.* 1989), and mechanical crushing (Grounds and McGeachie, 1992). In experiments designed to study the regenerative capacity of skeletal muscle, several different chemicals were used, including bupivacaine (marcaine) (Sadeh, 1988; Benoit and Belt, 1970; Sadeh *et al.* 1985; Hall-Craggs and Singh Seyan, 1975) and notexin (Whalen *et al.* 1990; Davis *et al.* 1989). From these studies, a clearer understanding of the muscle regeneration process has been established.

1.5.1 Muscle Degeneration Followed by Regeneration

Upon muscle injury and degeneration, muscle fibres will undergo a series of repair processes characteristic of the regeneration process. Satellite cells within the area re-enter the cell cycle and differentiate into myoblasts, which fuse and elongate to form immature myotubes. Along with innervation, these myotubes will then grow circumferentially, and with the fusion of other myoblasts will grow into mature fibres (reviewed by Grounds, 1991). Migration of the nuclei to the fibre periphery, and differentiation of fibre type finish the regeneration process (Allbrook, 1981).

Many different factors affect the rate and extent of muscle fibre regeneration, including the type and extent of the original damage (McGeachie and Grounds, 1987), the type of muscle fibre affected (Benoit and Belt, 1970) and the environment in which the regeneration takes place (Hall-Craggs, 1974; Carlson, 1973). The one absolute requirement for muscle regeneration is the presence of at least part of the muscle that has been affected (Bischoff, 1974). The essential part to be maintained

is the basal lamina, in which the satellite cells are located (Mauro, 1961). Once the muscle begins to degenerate, satellite cells are stimulated to re-enter the cell cycle and their progeny will then develop into either satellite cells or muscle precursor cells (myoblasts) (Allbrook, 1962). Following the conversion of satellite cells to myoblasts, these cells will then fuse to form myotubes. The fusion process is different from that previously described for the development of 1° and 2° myoblasts in which only myoblast-myotube fusion occurs (Duxson *et al.* 1989). In regeneration, researchers have also observed myotube - myotube fusion in addition to myoblast - myotube fusion (Jirmanova and Thesleff, 1972). At the same time that fusion is taking place, innervation is also occurring and these new myotubes are very susceptible to acetylcholine (Jirmanova and Thesleff, 1972). At this point, nuclei are still within the centre of the fibres, but with time, these will migrate once again to the periphery and fibre type will again be established (Hall-Craggs and Singh Seyan, 1975). The presence of central nuclei has been used as a marker to show that degeneration of a fibre has occurred (Allbrook, 1981). Myofibril patterns can be detected very early within the regenerative stage and these patterns become more developed as regeneration continues (Allbrook, 1962). In many cases, the final regenerated muscle contains higher amounts of fat and connective tissue than normal muscles indicating that damage to the basal lamina was too severe and support to the regenerating muscle was insufficient (reviewed by Allbrook, 1981)). This accumulation is not found when myotoxic drugs were used (Benoit and Belt, 1970).

1.5.2 Induced Degeneration of Skeletal Muscle by Marcaine (Bupivacaine)

Several different toxins and chemical agents have been used to target muscle degeneration (Hall-Craggs and Singh Seyan, 1975; Whalen *et al.* 1990). These chemicals cause a very rapid degeneration followed by an equally rapid recovery. One of these agents is bupivacaine (marcaine), which is a local anesthetic, and causes degeneration without damage to the nerve supply, blood supply or basal lamina (Benoit and Belt, 1970). Marcaine (bupivicaine or 1-n-butyl-DL-piperidine-2-

carboxylic acid-2,6-dimethyl anilide hydrochloride) has been used to examine the process of regeneration and its affect in neuromuscular diseases (Hall-Craggs, 1974; Hall-Craggs and Singh Seyan, 1975; Libelius *et al.* 1970; Sadeh, 1988; Sadeh *et al.* 1985), thereby providing a better understanding of disease related symptoms.

Notexin, a snake toxin, also causes muscle necrosis and degeneration since it contains phospholipase A_2 activity, a known myotoxic agent (Whalen *et al.* 1990). Several anaesthetics can also cause local muscle damage, such as lidocaine (diethylaminoacet-2,6-xylidide) (Smith *et al.* 1969). With marcaine, it appears that only the mature muscle fibres are the targets of degeneration. Repeated injections, on successive days into the same area, produced only new waves of degeneration, while differentiating myotubes were relatively unaffected (Benoit and Belt, 1970).

When marcaine is administered, the muscle fibres in the surrounding area degenerate within the first fifteen minutes after injection (Benoit and Belt, 1970). Within two days, the degeneration process is quite extensive and macrophages have entered the area. Nuclei migrate to the centre of the degenerating fibres and succinic dehydrogenase activity decreases dramatically (Smith, 1965). Satellite cells have also begun to divide and differentiate into myoblasts, with myotube formation being seen as early as day three (Hall-Craggs and Singh Seyan, 1975). Polygonal - shaped fibres can be seen within a week after injection. Further maturation and differentiation, both phenotypically and histochemically, is usually complete within a month (Benoit and Belt, 1970). The regenerated fibres appear to be similar to that of non - injected muscles within the same rat (Sadeh, 1988). This process may vary due to several internal factors. The age of the rat affects the rate and extent of regeneration, with older rats (~ 2 years of age) not being able to complete the regenerative process (Sadeh, 1988). The phenotype of the affected fibre also seems to cause some differential affects, with the larger, white fibres showing decreased susceptibility to the marcaine, as well as IIB fibres being more susceptible than IIA (Benoit and Belt, 1970). This fibre - specificity is also found to occur with denervated muscles, in which type I fibres showed higher susceptibility to

degeneration than type II fibres, while IIA fibres are more resistant than IIB fibres (Jakubiec-Puka *et al.* 1990).

There is still some controversy as to the mechanism by which marcaine causes specific degeneration of muscle fibres. Some local anaesthetics have been known to inhibit ATPase activity, but this has not been found to be the case for marcaine (Libelius *et al.* 1970). Libelius et al (1970) suggest that marcaine affects the mitochondria, interfering with the activity of its oxidative enzymes, such as succinic dehydrogenase. This would explain the muscle fibre specificity of the degeneration. A second theory is that marcaine causes temporary vasoconstriction followed by ischemia, thereby affecting the blood and oxygen supply to the muscles (Hall-Craggs, 1974). Slow fibres would therefore be preferentially affected due to the higher blood supply they receive. Once again, this would not explain the specific effects on muscle fibres, with no effect on myoblasts or myotubes. A third theory suggests that marcaine possibly competes against Ca^{2+} - binding membranes such as the sarcoplasmic reticulum (Cox and Gunter, 1973). Because muscle fibres depend greatly on the ability to bind calcium, the fibres would degenerate when this process was blocked. Although myoblasts and myotubes would also be affected, they would not degenerate because they are not as dependent on calcium - binding membranes (Allbrook, 1981). Although the method of action has not been elucidated, researchers have determined how to increase the effects of marcaine. By co-injecting hyaluronidase along with marcaine, the degeneration can become much more widespread (Hall-Craggs, 1974). Hyaluronidase degrades hyaluronic acid which is involved in the collagen fibre structural framework. This disruption allows the marcaine to diffuse more readily throughout the muscle (Hall-Craggs, 1974).

Through the use of marcaine and other myotoxic drugs, the effects of continual degeneration and regeneration have been examined and compared to symptoms of neuromuscular disorders, such as Duchenne Muscular Dystrophy (Grounds and McGeachie, 1992; Grounds *et al.* 1992). Central nucleation and longitudinal splitting of fibres were originally believed to be a direct result of several

such diseases. However, after exposing muscles to marcaine for several weeks or even months, this was not found to be the case (Sadeh *et al.* 1985). Repeated rounds of degeneration followed by regeneration prevented fibres from regenerating completly (Sadeh *et al.* 1985). Longitudinal splitting appears to result from the fact that the regenerating fibres fail to fuse with the basal lamina that is present from the previous fibre. This results in several fibres, with similar phenotypes, clustered together (Swash and Schwartz, 1977).

These regeneration experiments have also been used to examine the actual regenerative pattern itself, and how closely it parallels original muscle development. Although the fusion process is different from 2° myotube development (Jirmanova and Thesleff, 1972), the other aspects of regeneration are quite similar. Although it has been established that the original fibre-type pattern returns to normal after approximately four weeks of regeneration (Benoit and Belt, 1970), the actual regenerative pathway of MyHC expression has not yet been determined for marcaine induced regeneration. The MyHC pattern has been established for regeneration after notexin damage, which has a repair process similar to marcaine. Initially, all regenerating fibres had a characteristic expression of embryonic and neonatal MyHC (Whalen *et al.* 1990). Depending on the muscle that is regenerating, the effects of innervation may or may not cause a switch in phenotype. For example, it was observed that, in the soleus, this early expression was gradually replaced by slow-type MyHC. However, all the fibres became slow, instead of resulting in the original pattern of mixed fibre types (Whalen *et al.* 1990). Other muscles showed the characteristic mosaic of fibres (Carraro *et al.* 1983). If the regenerating soleus was denervated, however, only fast type fibres resulted, suggesting that innervation affected the pattern of MyHC expression during regeneration (Whalen *et al.* 1990). Supporting evidence showed that if innervation was re-established after 20 days, slow expression would again eventually predominate (Davis *et al.* 1989). These results suggested that slow MyHC maintenance was directly dependent on innervation, in agreement with several other research groups (Gorza *et al.* 1988;

Rubinstein and Kelly, 1978; Condon *et al.* 1990).

1.6 *In Vitro* Analysis of Cell Lines and Primary Myoblasts

Since it is more difficult to analyze the effects of different environmental factors *in vivo*, myoblast growth and differentiation have been examined *in vitro* (Smith and Miller, 1992; Stockdale and Miller, 1987). These cultures may consist of primary cultures in which the cells are derived directly from the animal (Vivarelli *et al.* 1988; Smith and Miller, 1992; Stockdale and Miller, 1987; Yaffe and Saxel, 1977; Mulle *et al.* 1988), or myoblast cell lines, which have been established from several different organisms including birds (Antin and Ordahl, 1991), mice (Yaffe and Saxel, 1977) and rats (Yaffe, 1968). These cell lines provide a stable population of myoblasts that will differentiate and fuse in the proper conditions, while primary cell lines are not very stable and will not last beyond several passages in culture. These cells lines have been used to study the controls and determining factors for muscle development (Muthuchamy *et al.* 1992; Pajak *et al.* 1991).

1.6.1 L6 Myoblasts

The most widely used rat myoblast cell line is the L6 line, which was established from neonatal cell cultures by Yaffe (Yaffe, 1968). This cell line has a very characteristic pattern of MyHC expression in which only embryonic myosin heavy chain is observed upon differentiation in culture (Wieczorek *et al.* 1985). Recently, it has been reported that L6 cells also produce mRNA transcripts corresponding to neonatal, fast IIA and slow MyHC (Muthuchamy *et al.* 1992). However, there is no evidence of any production of the corresponding proteins for these transcripts.

The mrf expression of this line is also interesting in that there is no production of MyoD (Hinterberger *et al.* 1991; Wright *et al.* 1989) during differentiation in culture. This is remarkable since these cells will grow, differentiate and fuse without this MyoD expression. This may indicate one of two possibilities. First, it may be

that MyoD is not needed for the fusion and differentiation of muscle cells to take place. Alternatively, it could be that these cells have the ability to compensate for the lack of MyoD with other endogenous factors. This second hypothesis is supported by evidence which shows that myf-5 is expressed within L6 cells, unlike other cell lines in which myf-5 is not detected (Hinterberger *et al.* 1991). The fact that the L6 cell line does not express certain mrfs allows it to be an excellent vehicle for analyzing the roles of the different mrfs in muscle development. For example, it has been shown that exogenous production of MyoD turns on the cardiac α-actin and myosin light chain 1 transcripts, which are not usually expressed in L6 myotubes (Muthuchamy *et al.* 1992). L6 nuclei can also be induced to express myosin light chain 1 by fusing them with C2C12 cells (Pajak *et al.* 1991), which are derived from adult mice (Yaffe and Saxel, 1977) and are known to express MyoD. These results indicate a possible function for the MyoD protein in muscle development.

1.6.2 Myosin Heavy Chain Expression in Primary Cell Lines

Primary cell populations of hindlimb skeletal muscle have been examined to help determine if all muscle cells are phenotypically similar. Through mass cultures of specific developmental time points and clonal analysis within the same time frame, investigators have determined that all muscle cells do not appear to be equal. By analyzing either the MyHC expression (Vivarelli *et al.* 1988) or the response of various myoblast populations to environmental changes (Cusella-De Angelis *et al.* 1994), it has been shown that the muscle populations may indeed be different.

Vivarelli et al (1988) suggest that there are at least two different myoblast populations, separated by time, involved in muscle development in the mouse hindlimb. This was determined by growing pure populations of hindlimb skeletal mouse cells, obtained from day 10 and 15 mouse fetuses. After five days in culture, the early myoblasts, which were obtained from the hindlimb buds of the embryo, were uniform in their expression of MyHC isoforms. All myotubes expressed both

embryonic and ß- cardiac (slow - type) myosin. The older myoblasts, obtained from hindlimb muscles, also expressed an embryonic MyHC isoform, along with neonatal and adult fast isoforms. No slow isoform was expressed until 20 days in culture, at which point only 5% of the myotubes expressed the slow isoform. This seems to be in direct correlation to the *in vivo* results in rats discussed earlier (Condon *et al.* 1990), and would suggest that there are basically two separate populations of myoblasts, separated by time within the developing hindlimb. These two populations have been referred to as "embryonic" and "fetal" myoblasts.

In a similar study, Smith and Miller (Smith and Miller, 1992) contradicted this pattern of MyHC expression by showing the presence of slow MyHC in the later (fetal) cultures and the presence of neonatal in the earlier (embryonic) cultures. The slow isoform was observed in fetal cultures after only five days in culture, unlike the earlier studies, in which slow MyHC was only detected at day 20 (Vivarelli *et al.* 1988). The neonatal isoform was only present in a very small subset of myotubes (~ 5%) in the embryonic cultures, and the staining was never very intense. These results by Miller suggest that there are several different populations of myoblasts within the mouse hindlimb at any one stage of development. The embryonic cultures showed three different phenotypes in culture; one with embryonic expression only, one with embryonic and slow expression, and a third that expressed embryonic, slow and neonatal MyHCs (Smith and Miller, 1992). There was no expression of adult fast isoforms, and neonatal MyHC was never expressed without the expression of slow MyHC. The fetal cultures showed two possible populations; one expressed neonatal MyHC and the other expressed slow MyHC. Both populations expressed embryonic MyHC as well. Based on the above studies it would seem that there are definitely two different populations within the mouse hindlimb during development of the muscle. Whether or not these populations represent myogenic lineages or are divided into smaller sub-populations has not been fully determined.

1.7 Therapeutic and Experimental Use of Myoblast Transplantation

Myoblast transplantation was first proposed as a therapy to treat muscle - specific genetic disorders (Partridge and Sloper, 1977). The technique involves the injection of myoblasts into an area where they may fuse to host muscle tissue and, through maintained expression of their protein profile, alter or add to the host fibres' gene expression (reviewed by Karpati, 1990; Karpati *et al.* 1993; Pagel and Morgan, 1995; Partridge, 1991). In the case of diseased muscles (eg. Duchenne Muscular Dystrophy (DMD)), normal donor myoblasts would fuse to genetically abnormal cells and the resulting muscle fibres would express the protein(s) that had been missing. Several different strains of mice, such as the dy^{2J} mouse (Law *et al.* 1988; Law *et al.* 1988) and the mdx mouse (Morgan *et al.* 1989; Karpati *et al.* 1989; Karpati *et al.* 1989; Wakeford *et al.* 1991; Morgan *et al.* 1990) have been used to address the different problems in the procedure.

1.7.1 Experimental Parameters of Myoblast Transplantation

Several key issues need to be addressed when using myoblast transplantation as an experimental technique, or as a therapeutic tool. First, culture conditions must be established in which large quantities of myoblasts can be obtained (reviewed by Karpati, 1991). Although satellite cells can be grown in culture for long periods of time, there are limitations on the number of passages they can survive before they lose their ability to differentiate. It is also possible to use stable cell lines in which numbers of myoblasts would not pose a problem (Morgan *et al.* 1992). However, several studies injecting such cell lines into mice have reported the formation of tumours (Wernig *et al.* 1991; Rando and Blau, 1994). Recently, it has been suggested that fibroblasts may represent a potential source of myogenic cells (Breton *et al.* 1995; Gibson *et al.* 1995). Although it is possible that these cells may be converted into myoblasts, it still seems unlikely that they would provide the numbers necessary for large scale myoblast transplantation.

A second obstacle that must be overcome when performing myoblast

transplantations is how to develop a method for tracing the donor cells *in vivo*. In the case of myogenic disorders, such as DMD, it has been possible to follow the expression of the protein that is deficient in the host, but not the donors (Morgan and Watt, 1993; Karpati, 1990; Law *et al.* 1993). An alternative to this approach has been to label cells *in vitro* with either cytoplasmic (Price *et al.* 1987) or nuclear (Labrecque *et al.* 1991) markers. Several criteria are necessary to make a marker ideal for tracing cells. First, the marker must not be diluted out by repeated divisions. Secondly, in experiments where the fate of individual nuclei is being examined, it is desirable for the marker to localize specifically to the donor nuclei or perinuclear area. Finally, it is important for the marker to be easily detected.

Several different markers have been proposed for myoblast transplantation. However, no one marker has proven ideal. The use of thymidine analogues such as 5'-bromodeoxyuridine (BRDU) (Labrecque *et al.* 1991) and radioactive labels ([H^3]Tdr) (Karpati *et al.* 1989) can be specifically localized within the donor nucleus. However, they are quickly diluted out by cell division and often not easily detected. The use of the Barr body (Beilharz *et al.* 1992) or Y-chromosome genes (Grounds *et al.* 1991) have also been suggested. These would show stable inheritance from one cell generation to the next, but would pose problems in the positive identification of donor nuclei, since sections of nuclei which do not contain these markers would often be observed. Also, probes for these markers are available for only a limited number of species, and are not available for the rat.

The most commonly used marker to date is a retrovirus containing two genes (Price *et al.* 1987). The first is the lac-Z gene which allows for the specific identification of fibres containing donor nuclei. The second gene is one that provides resistance to neomycin, and is used to positively select cells in culture (Price, 1987). The lac-Z gene produces the β-galactosidase enzyme (β-gal) which reacts with X-gal to produce a traceable blue colour (Shimohama *et al.* 1989). The genes are stably inherited by daughter cells and are easy to trace. However, due to diffusion, β-gal will spread through the fibre, making it difficult to localize the specific donor nucleus

(Ralston and Hall, 1989). Nuclear targeted β-gal has a smaller range of diffusion, but will label host nuclei since the nuclear targeting is non-specific (Ralston and Hall, 1989).

The third parameter that must be dealt with for myoblast transplantation is the receptiveness of the host to the graft. Initial studies showed that syngeneic hosts did not need any immunosuppression for the maintenance of the graft (Rando and Blau, 1994). There is now strong evidence to suggest otherwise. Experiments have shown that rejection of muscle grafts does occur (Labrecque *et al.* 1992; Huard *et al.* 1992). Different immunosuppressants, including cyclosporin (Labrecque *et al.* 1992) and FK506 (Kinoshita *et al.* 1994), have been used extensively with varying results. The most recent evidence indicates that only transient immunosuppression is necessary for the maintenance of the graft, suggesting that the graft will lose its susceptibility to rejection over time (Watt *et al.* 1984; Pavlath *et al.* 1994).

1.7.2 Basic Research Using Myoblast Injection Therapy

Although the majority of the research using myoblast injection therapy involves the development of a technique to combat hereditary muscle disorders (Karpati *et al.* 1993; Law *et al.* 1993; Gussoni *et al.* 1992), the technique itself has been used as a research tool to answer more basic scientific questions. Analysis of muscle cell lineage (Hughes and Blau, 1992) and migration of myoblasts between muscles (Hughes and Blau, 1990; Watt *et al.* 1987) has been examined to some extent. Although the results are preliminary, they are beginning to provide a clearer understanding of muscle development and repair. With the use of markers, such as retroviruses, it is now becoming possible to analyze the fate of myoblasts in individual fibres (Price, 1987).

For years there has been controversy over the repair process of muscle following localized damage to individual fibres. At first it was believed that muscles repaired themselves by internal nuclear divisions and growth (Lash *et al.* 1957).

However, with the discovery of satellite cells (see above), it was determined that the regeneration was due to fusion of new muscle precursor cells with myotubes or with each other (Mauro, 1961). Blau and coworkers showed that the satellite cells not only fused with the fibre that it was associated with, but also migrated out of the fibre and fused with other muscle fibres (Hughes and Blau, 1990). These results supported work done previously, in which donor grafts were infiltrated by host myoblasts from adjacent areas (Partridge and Sloper, 1977; Watt *et al.* 1987). Now that an established technique has been developed to introduce foreign myoblasts into an area, researchers have started to examine the fate and expression of different myoblast cell lines *in vivo*. To trace these cells, myoblasts have been infected with a retrovirus containing the β - gal and neomycin resistance genes (Hughes and Blau, 1992), or other reporter genes (DiMario *et al.* 1993; DiMario and Stockdale, 1995).

Upon injection of donor myoblasts, two possible types of fusion may occur. Fusion of donor cells to host cells can result in heterotypic fibres, in which nuclei of different origins share the same cytoplasm. Alternatively, donor myoblasts may fuse with themselves, resulting in homotypic fibres which only contain the nuclei of one myoblast population. Homotypic fibres have been reported by Stockdale's lab (DiMario *et al.* 1993; DiMario and Stockdale, 1995) who injected primary quail myoblasts of both fast and slow origin into the hindlimb buds of five day old chick embryos. These fibres exhibited complete maintenance of the *in vitro* MyHC expression characteristic of the donor cells regardless of which muscle they were injected into. However, the time period examined did not allow for the analysis of long term environmental effects on the phenotype, and the myotubes were not analyzed for their state of innervation. Another study has revealed that such myotubes can become innervated, starting at four weeks post-injection (Wernig *et al.* 1991). These studies based their conclusions on the localization of acetylcholine receptors and NCAM within the muscle fibres. Although muscle fibre types were not carefully analyzed and the *in vitro* phenotype of the donor myoblasts was not determined, a general transition of slow to fast fibres occurred beginning at four

weeks post-injection. It was uncertain whether these two events represented a causal relationship.

The fate of donor nuclei within heterotypic fibres has also been examined. In this study, the fate of a mouse cell line (C2C12) or mouse satellite cells were analyzed (Hughes and Blau, 1992). Clones of myoblasts derived from either fast or slow muscles were injected into the hindlimb muscle of adult mice and their developmental potential was examined up to 38 days post-injection. These experiments showed that the injected cells lost their *in vitro* phenotype and adopted the phenotype of the fibres with which they fused. Unfortunately, very few fibres could be observed which contained donor nuclei and it was likely that the donor nuclei were greatly outnumbered by the host nuclei within each fibre. The possibility that the donor nuclei were being overwhelmed or even "turned off" by potential trans-acting factors from host nuclei was not addressed. Also, the *in vitro* MyHC profiles of the injected cell populations included the expression of a vast array of isoforms, indicating that the cells may be able to adapt to all environments (Düsterhöft and Pette, 1993). Finally, labelling of the satellite cells required extensive culturing which may have affected any intrinsic programming of the cells (Karpati, 1991).

1.8 Factors Influencing the Development of Muscle Fibre Types

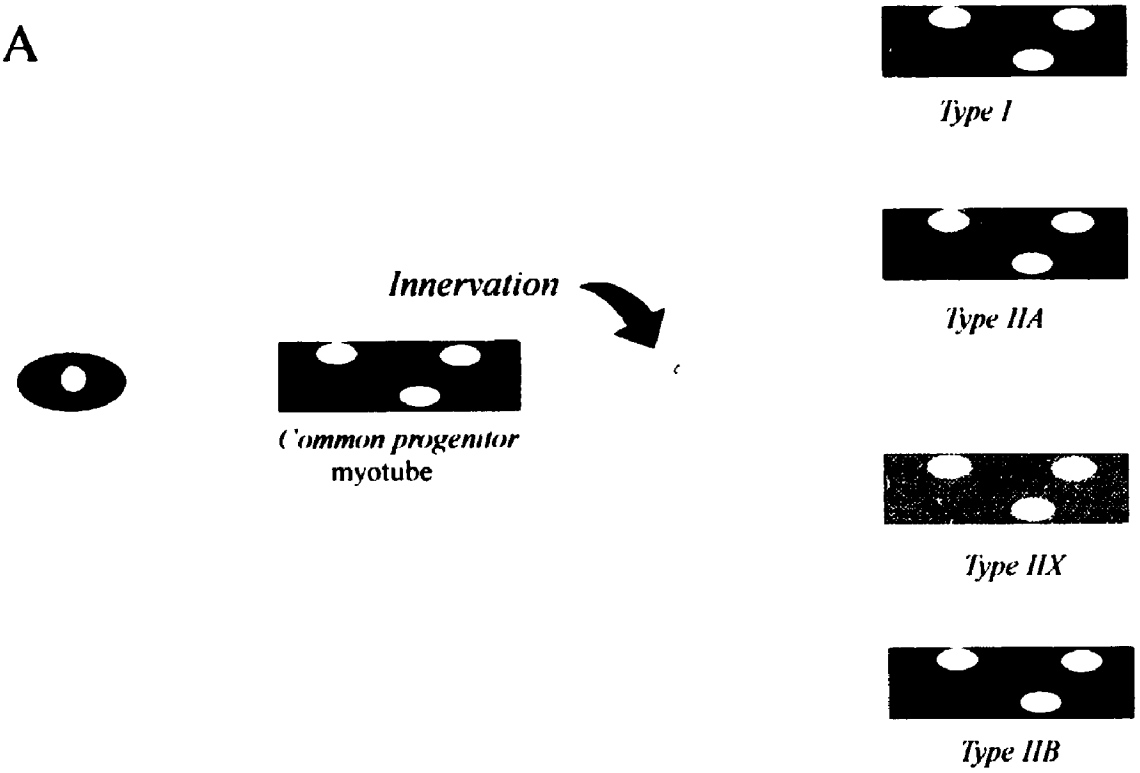
The factors involved in establishing muscle fibre types can be divided into two general classes, extrinsic (environmental) and intrinsic. Two simplified models have been proposed which suggest that the development of muscle fibre types is controlled by only one class of factors (Figure 1.1). The first model proposes that environmental cues (specifically innervation) are the only determining factors involved. Inherent to this model is the premise that only one myogenic lineage exists, and this is the common progenitor for all fibre types. All fibre types are established after fusion and are strictly controlled by the state of innervation. The second model proposes that each fibre type develops from individual myogenic lin-

Figure 1.1 Proposed models describing the development of different muscle fibre types.

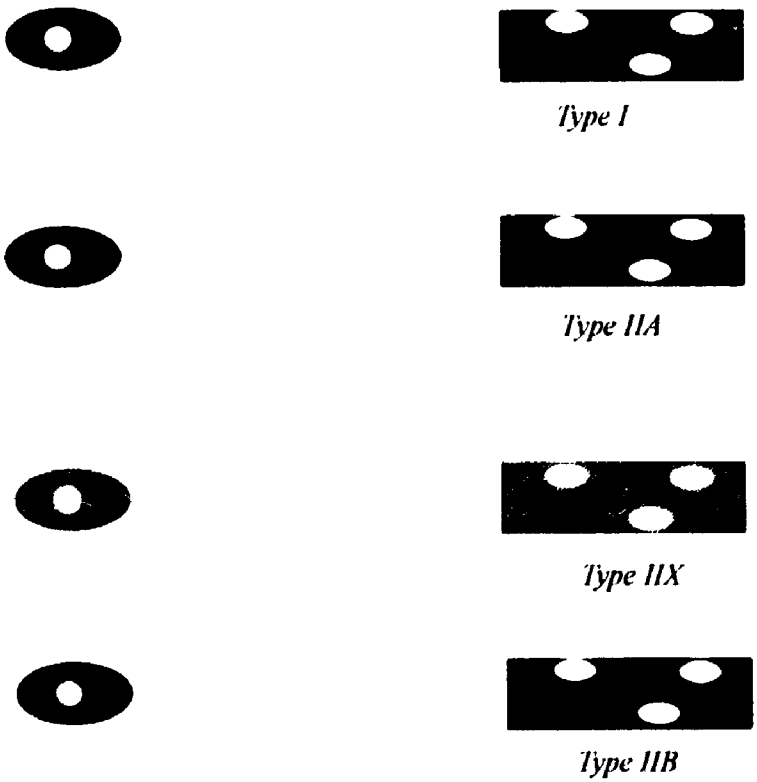
(A) All myotubes are derived from a common myoblast population. Following innervation, these myotubes mature and differentiate into the various adult fibre types, which are slow (I) and fast IIA, IIB and IIX. Therefore development of all fibre types is governed by external cues.

(B) Each muscle fibre type develops from a corresponding myoblast population. Therefore, four myogenic lineages exist and the MyHC phenotype of individual fibres is governed exclusively by intrinsic factors.

A



B



Fibre types develop in the absence of innervation

eages. Inherent to this model is the idea that muscle phenotype is established prior to differentiation and that environmental cues play no role in the initial formation of the different fibre types.

The effects of innervation on final fibre type expression has been demonstrated through experiments which alter the normal innervation patterns of the adult fibre. By changing the activity patterns of the nerve (Gorza *et al.* 1988) or innervating fibres with different motoneurons (Buller *et al.* 1960), the original fibre type can be changed to one appropriate to the innervation pattern being imposed. The nerve-dependent induction of fibre type has not been observed during muscle development. 1° fibres, which initially develop without innervation, show normal patterns of expression in aneural fetal muscles (Condon *et al.* 1990). This contradicts Rubinstein and Kelly (1978), who suggested that the development of slow - type fibres is dependent on nerve stimulation. These researchers may be partially correct, since it appears that there is a decrease of slow 1° myotubes following denervation, especially in the soleus (Narusawa *et al.* 1987), and there is no switching of 2° myotubes to a slow - type expression. Condon et al (1990) also suggest that innervation may only be necessary for the maintenance of the slow phenotype. It has also been suggested that there are different types of slow fibres, with one group being nerve - dependent and the other group being nerve independent (Narusawa *et al.* 1987). In denervated muscle, the transition of slow MyHC to neonatal MyHC is also affected since these two isoforms are not co-expressed, but rather, the slow isoform disappears, and then the neonatal myosin appears one day later. This lack of co-expression is due to an overall delay of neonatal myosin expression in aneural fetal hindlimbs (Condon *et al.* 1990). Narusawa et al (1987) suggest that all transitions are the result of innervation, and, therefore, no 1° myotube should express neonatal myosin. Reports have shown that this is not true, and that transitions may occur without the presence of innervation, at least in the case of changes of slow myosin to fast myosin (Harris *et al.* 1989; Condon *et al.* 1990).

Another controversial issue is that of 2° myotube development in aneural muscles. As previously mentioned, it was originally believed that these fibres did not form in denervated fetuses (Wilson *et al.* 1988; Duxson *et al.* 1989; Sheard *et al.* 1991). However, this was shown to be false, and the 2° myotubes that do develop maintain the normal phenotype with the exception that there is no switch of phenotype to slow fibres (Condon *et al.* 1990). *In vitro* studies also contradict the theory that innervation is necessary for 2° myotube formation, by showing that the phenotypes seen *in vivo* are maintained in culture without the presence of nerve interaction in other animal models such as chicken (Stockdale and Miller, 1987) and mice (Vivarelli *et al.* 1988). Specifically, populations of myoblasts derived from early time points in gestation exhibit slow MyHC phenotypes while later populations exhibit fast MyHC phenotypes. These observations indicate that there may be some intrinsic qualities to the myoblast populations forming 1° and 2° fibres. Therefore, these myoblast populations may represent myogenic lineages which have a predetermined developmental potential (Stockdale, 1992). However, it should be noted that in recent studies examining similar populations of myoblasts in human, no differences in their developmental potential *in vitro* were observed, suggesting that such lineages do not exist in humans (Cho *et al.* 1993). Currently, no such characterization has been performed for the different populations in rat development.

In examining the role of other environmental influences on muscle phenotype, three general approaches have been used. First, studies were performed in which the electrical stimulation patterns of muscles were altered. This involved cross-innervation experiments in which predominantly slow muscles were denervated and then re-innervated with a typically fast nerve, or vice versa. Such cross-innervation experiments resulted in a transition of the re-innervated muscle fibres appropriate to the specific pattern of stimulation (Buller *et al.* 1960; Eldridge *et al.* 1984). Other experiments which involved the direct stimulation of muscle fibres with patterns of electrical activity similar to fast innervation produced similar results, where transitions from a slow to fast phenotype were observed (Gorza *et al.* 1988; Pette and

Düsterhöft, 1992; Termin *et al.* 1989). Secondly, the muscle environment was altered through changes in hormonal or trophic influences present on the fibres. Examples of such changes included increases in the levels of thyroid hormone which produced a switch in fibre type to faster phenotypes (Izumo *et al.* 1986; Gambke *et al.* 1983; Butler-Browne *et al.* 1984). Finally, myoblasts labelled with markers to allow their tracing *in vivo*, were injected into various muscle environments and then analyzed for their expression. Fusion of individually labelled myoblasts onto adult host fibres resulted in MyHC expression typical of the host fibre (Hughes and Blau, 1992). All of these studies have indicated that final muscle phenotypes are regulated strictly by environmental influences.

Although all of the above approaches were interpreted to suggest that extrinsic factors determined the final muscle phenotype, related experiments have provided evidence for the existence of intrinsic controls as well. In some cross-innervation studies, it was observed that a population of fibres consistently remained refractile to the changes in innervation (Thomas and Ranatunga, 1993; Graybiel, 1990). A similar population of fibres also appeared to be resistant to change following treatment with increased levels of thyroid hormone (Fitzsimons *et al.* 1990). These findings indicated that in each case a subset of muscle fibres did not respond to the environmental changes presented and may be governed more tightly by intrinsic factors. Also, injection of myoblasts into developing muscle produced substantially different results from the injections of myoblasts into adult muscles since donor myoblasts in developing muscle maintained their *in vitro* phenotype (DiMario *et al.* 1993; DiMario and Stockdale, 1995). Even though the differences between the injected cells or the experimental designs may account for the conflicting observations, the fact remains that there is now a growing body of evidence to suggest that both intrinsic and extrinsic factors contribute to phenotypes in muscle.

1.9 Objectives

The purpose of this study was to examine different populations of rat myoblasts to determine if (a) separate myogenic lineages existed with specific defined phenotypes, (b) when such lineages are established, and (c) how these myogenic lineages are affected by environmental factors. The working hypothesis for this study was that different myoblast populations represent distinct myogenic lineages with distinct developmental potentials. To address this hypothesis, it was first necessary to determine the phenotype of myoblasts obtained from different gestational time points (Chapter 2) or of L6 rat myoblasts (L6 myoblasts) in culture. To do this, a panel of monoclonal antibodies was established to identify all of the individual members of the MyHC family, and then used to characterize the three myoblast populations. Once definite phenotypes were established, these populations were then placed in various environments to test the stability of that phenotype. First, to determine if the phenotypic profile of the myoblasts was established prior to fusion, cells derived from ED 14 and ED 20 rat hindlimb were independently labelled and then co-cultured (Chapter 3). These co-cultures were analyzed to determine if the two populations fused with each other to form muscle heterokaryons and, if they did, what the phenotype of the resulting myotubes would be. This experiment would also allow us to determine if *cis*- or *trans*-acting factors were involved in the programming of individual nuclei. Next, ED 14 and ED 20 cell populations were injected into the caudate-putamen of adult rats. This approach had a dual purpose. First, it was possible to examine the two populations for a longer period of time than could be followed *in vitro*. Secondly, the cells were placed in a permissive environment that produced both neurotrophic and ubiquitous growth factors reported to be involved in the full maturation of the muscle (Chapter 4). This experimental design allowed for the examination of the long term fate of the cells and helped determine if local cues would alter their fate. The last approach was to trace labelled L6 myoblasts after injection into regenerating muscle. By analyzing the fate of these cells after fusion with themselves (Chapter 6) or host tissue (Chapter

7), it was possible to determine if the phenotype of these cells was maintained within various slow and fast environments. Through the experiments outlined here, it was possible to address the question of whether different myogenic lineages exist in which their final phenotype is governed in part by intrinsic mechanisms.

CHAPTER 2 - CHARACTERIZATION OF EMBRYONIC AND FETAL MYOBLASTS *IN VITRO*

2.1 Introduction

During development of the rat hindlimb *in utero*, skeletal muscles develop in two distinct stages. The first differentiated muscle cells - or primary (1°) myotubes - begin to appear in rat hindlimb muscles on embryonic day 14 (ED 14) (Condon *et al.* 1990) and reach a stable number by ED 17 (Ross *et al.* 1987). Within 48 hrs, a second population of myotubes - secondary (2°) myotubes - forms on the surface of the 1° myotubes beneath the basal lamina, and uses the 1° myotubes as a scaffolding for their subsequent development (Kelly and Zacks, 1969; Harris *et al.* 1989). In addition to this difference in temporal appearance, 1° and 2° myotubes can also be distinguished by differences in morphology, since 2° myotubes initially have a smaller diameter and appear around the periphery of centrally located 1° myotubes (Ross *et al.* 1987). Most importantly, 1° and 2° myotubes also differ in their pattern of myosin heavy chain (MyHC) expression. Primary myotubes co-express both slow and embryonic (fast) MyHC, while 2° myotubes (at least initially) express only embryonic MyHC (Narusawa *et al.* 1987; Condon *et al.* 1990). This difference does not seem to be related to differences in innervation, since denervation does not alter the expression of MyHCs in these two distinct populations of myotubes (Condon *et al.* 1990). While 1° myotubes tend to retain their expression of slow MyHC and develop into adult Type I fibres, there are exceptions to this rule in some fast muscles, such as the extensor digitorum longus (EDL) and tibialis anterior (Narusawa *et al.* 1987; Condon *et al.* 1990). Secondary myotubes tend to sequentially replace the embryonic MyHC isoform with neonatal and then adult fast MyHCs to become Type II fibres, although some 2° myotubes in muscles such as the soleus can develop into slow, Type I fibres (Narusawa *et al.* 1987; Condon *et al.* 1990). Regardless of these exceptions, it is generally believed that the distribution of 1° and 2° myotubes in fetal muscles provides a "blueprint" for the pattern of fast and slow fibres in adult muscles. Thus, factors which regulate the differentiation and

survival of 1° and 2° myotubes - such as prenatal nutrition (Wilson *et al.* 1988) - can dramatically affect the normal fibre type of adult muscles.

At present, the biological basis for the differences observed between 1° and 2° myotubes is unknown. However, one theory suggests that these myotubes have different phenotypes and fates because they are derived from different populations of muscle-precursor cells (or myoblasts) (Stockdale, 1990; Stockdale, 1992). This theory is supported by the fact that 1° myotubes are generated by the fusion of myoblasts which appear early in limb development (i.e. around ED 13 - 14) while 2° myotubes are formed from myoblasts which populate the limb at later stages (i.e. ED 17-20; (Ross *et al.* 1987). Significantly, ultrastructural (Duxson *et al.* 1989) and myonuclear dating (Harris *et al.* 1989) experiments demonstrate that late myoblasts will only fuse with each other or nascent 2° myotubes and never with 1° myotubes, even though they may be juxtapositioned. This suggests that there are inherent differences between early and late populations of myoblasts which specify fusion-kinetics *in vivo* (Cossu and Molinaro, 1987). Differences between early and late classes of mouse myoblasts have also been described *in vitro* (Cossu *et al.* 1988; DeAngelis *et al.* 1992; Cusella-DeAngelis *et al.* 1992). Interestingly, Vivarelli et al (1988) and Smith and Miller (1992) have shown that, as in the chick (Miller *et al.* 1985; Stockdale and Miller, 1987), early and late murine myoblasts can be identified based upon differences in MyHC isoform expression following differentiation *in vitro*. Early (or embryonic) myoblasts form myotubes which co-express both embryonic (fast) and slow MyHC, while late (or fetal) stage myoblasts fuse *in vitro* to form myotubes which express embryonic and neonatal MyHC but not slow MyHC.

The objective of this study was to establish whether myoblasts prepared using similar methods from embryonic (ED 14) and fetal (ED 20) hindlimbs of the rat express similar or different phenotypes during differentiation *in vitro*. By examining myotube size and the fusion indices of the two myoblasts populations, we have shown morphological differences between the two populations of myoblasts. To

complete this study, a panel of monoclonal and polyclonal antibodies was used to examine MyHC expression in these myotubes. The antibodies used included embryonic (47A), adult fast IIA (SC.71 and 4A.74), IIB (BF.F3), IIB/IIX (212F), all neonatal / adult fast isoform (MY-32) and slow (10D10,4A9, 8H8, 4H3, and 4A.951) MyHC-specific Mabs, while polyclonal antibodies specific for the neonatal MyHC isoform (NN6) and desmin (AB907 and PPN.1101) were also used. The results demonstrate that embryonic and fetal rat myoblasts differentiate *in vitro* to form myotubes similar to the primary and secondary myotubes observed *in vivo*. Since these phenotypic differences were observed when both myoblast populations were cultured *in vitro* under similar conditions, it was concluded that embryonic and fetal myoblasts in the rat represent distinct myogenic lineages similar to those previously described in chickens (Stockdale and Miller, 1987) and mice (Vivarelli *et al.* 1988; Smith and Miller, 1992).

2.2 Materials and Methods

Unless otherwise stated, all chemicals were supplied by BDH Inc., Toronto, Ontario.

2.2.1 Characterization of MyHC-Specific Monoclonal Antibodies

Adult Wistar Furth rats (Harlan Sprague Dawley Indianapolis, IN) were sacrificed with a lethal injection of sodium pentobarbital (M.T.C. Pharmaceuticals, Cambridge, Ont.) and the plantaris muscles were dissected from the hindlimbs. These muscles were frozen in melting isopentane, embedded in Tissue Tek OCT (Miles Inc., Elkhart, In.) freezing compound. The resulting muscle blocks were serial sectioned at 10 - 15 µM onto gelatin chrom-alum slides (gelatin; Sigma Chemical Co., St Louis, MO, chrom-alum (chromium potassium sulfate); Fisher Scientific, Unionville, Ont.) using a Leitz cryostat and the sections were then stored at -20°C. To characterize the MyHC-specific Mabs using the Avidin-Biotin Complex (ABC) method (Hsu *et al.* 1981) in which biotinylated alkaline phosphatase formed part of the complex (ABC-AP), sections were fixed with 90% methanol for 6 min at

-20°C, blocked for 30 min with 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont.) in phosphate buffered saline (PBS) at 37°C and then incubated with the various antibodies for 1 hr at room temperature (RT). The various Mabs that were characterized are 47A (diluted at 1:10 in 0.1% BSA ([Gibco/BRL, Burlington, Ont.] in PBS), MY-32 (1:200), N1.551 (undiluted), 4A.74 (undiluted), SC.71 (undiluted), BF.F3 (undiluted), 212F (1:4), 4A9 (1:50), 10D10 (1:5), A4.840 (undiluted), 4A.951 (undiluted), 8H8 (1:50) and BF.35 (undiluted). Following incubation in the primary antibodies, sections were rinsed several times with PBS and incubated in a 1:1000 dilution of biotinylated rabbit anti-mouse (RAM) IgG-isotype specific antibodies (Dimension Labs, Mississuaga, Ont.) for one hr. Sections were rinsed again with PBS and incubated with the avidin-biotinylated alkaline phosphatase complex (ABC-AP; Dimension Labs, Mississauga, Ont.) for 1 hour at room temperature (RT). Following another set of rinses in PBS, the slides were incubated for 15-30 min at RT in substrate buffer (100 mM TRIS, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) containing 3.5 mg NBT, 2 mg BCIP (NBT,BCIP; Gibco/BRL, Burlington, Ont.) and 2.4 mg levamisol (Sigma Chemical Co., St Louis MO) per 10 mls. Slides were washed with PBS, coverslipped with aquamount and stored at 4°C until photographed.

2.2.2 Actomyosin ATPase Enzyme Histochemistry

As an independent measure of muscle fibre type, unfixed sections from the plantaris muscle were analyzed using an ATPase assay. Briefly, frozen sections were allowed to air dry for 30 min at RT and then submerged for 10 min into alkaline pre-incubation medium (50 mM glycine [Biorad, Mississauga, Ont]), 50 mM NaCl, 27 mM $CaCl_2$2HO, 0.1 N NaOH at pH 9.85) at 37°C. Sections were then rinsed 5 times with double-distilled water (ddH_2O) and placed in incubation medium (3 mM Na_2ATP [Sigma Chemical Co., St Louis, MO]), 85 mg/50 ml ATP-disodium salt crystalline at pH 9.42) for 30 min at 37°C. Sections were rinsed 5 times with ddH_2O and then incubated in 1.0% $CaCl_2$ for 3 min at RT. Sections were again rinsed 5

times followed by a 3 min incubation in 2% $CaCl_2$ at RT. After 5 more rinses, sections were placed in 1.0% $(NH_4)_2S$ for one min at RT, rinsed, dehydrated, mounted with DPX mountant, and dried overnight.

2.2.3 Primary Culture of Embryonic and Fetal Rat Hindlimb Myoblasts

Primary cultures were set up as described previously (Lawrence and Catterall, 1981) with some modifications. Timed pregnant Sprague-Dawely rats were obtained commercially (Charles River, Montreal, Que), where pregnancies were dated by the presence of a copulation plug the morning after coupling, as suggested by Harris et al. (1989). At the appropriate stage of gestation (ED 14 or ED 20), pregnant rats were heavily anaesthetized with 70 mg/kg body weight of sodium pentabarbitol and the fetuses were removed, dissected free of all membranes and transferred to a sterile tissue culture (TC) dish. Individual hindlimbs were dissected free at the hip joint and the skin and (in the case of ED 20 fetuses) femur removed. Hindlimb muscles from 10 - 40 fetuses were pooled in a 35 mm dish containing Ca^{++} Mg^{++} free Hanks Balanced Salt Solution (CMF-HBSS; Gib BRL, Burlington, Ont), drained and finely minc (100-200 times) with scissors. The mince was then dispersed in 20 mls of 0.25% trypsin (Gibco/BRL, Burlington, Ont) in CMF-HBSS / 15 fetuses, and incubated at 37°C for 30 minutes with agitation at 10 minute intervals. Cells were pelleted at 800 g for 10 minutes and resuspended in 10 mls of cold complete medium, consisting of 10% horse serum (HyClone Labs Inc, Logan, Utah), 5% fetal bovine serum (HyClone Labs Inc, Logan, Utah), 50 units/ml penicillin, 10 µg/ml streptomycin and 1.25 µg/ml, fungizone (psf; Gibco/BRL, Burlington, Ont) in 68% Dulbecco's Minimal Essential Medium (DMEM; Gibco/BRL, Burlington, Ont), and 17% Medium 199 (Gibco/BRL, Burlington, Ont). Chunks of tissue were allowed to settle for 2 - 3 minutes and the cell suspension was then passed through a 40 µM Nitex filter (Millipore Corp., Bedford, MA) and an aliquot counted on a haemocytometer. Primary plates were set up on 100 mm Falcon TC dishes (Canlab Scientific Products, Mississauga, Ont) precoated with 100 µg/ml gelatin (Sigma

Chemical Co., St Louis, MO) as prescribed by Konigsberg (1979), at a density of 1 x 10^7 cells/ 100 mm dish and incubated overnight in a 37°C incubator containing 5% CO_2.

2.2.4 Selective Trypsinization of Primary Cultures to Enrich for Myoblasts

This method for enriching for myoblasts exploits the differential attachment of myoblasts and fibroblasts to the gelatin-substrate, and was carried out essentially as described by Konigsberg (1979). Primary cultures were rinsed twice with CMF-HBSS and incubated with 2.5 mls of a 200 µg/ml solution of crystalline trypsin (Gibco/BRL, Burlington, Ont) in CMF-HBSS for 3-5 min until approximately 10% of the cells (mostly myoblasts) were released from the substrate. Cells were immediately added to 0.5 mls (per plate) of complete medium containing 0.5 mg/ml soybean trypsin-inhibitor (Gibco/BRL, Burlington, Ont), and counted in a haemocytometer. Mass cultures were set up in 4 well chamber slides (Gibco/BRL, Burlington, Ont) at 1.55 x 10^5 cells/well; in 6 well plates at 1 x 10^6 cells/well and at 1 x 10^7 cells/ 100 mm dish. Cytosine arabinoside (ARA-C; Sigma Chemical Co., St. Louis, MO) was added to the mass cultures at a final concentration of 10 µM at day 4 of incubation for 48 hrs, then the cultures were refed with complete medium and incubated for up to 8 more days. Plates were fixed with 90% methanol at -20°C for 6 minutes for immunolocalization studies at 1, 2, 3, 4, 6, 8 and 10 days after selective trypsinization and replating.

2.2.5 Immunolocalization of MyHC Isoforms in Myoblast Cultures

To examine the accumulation of various MyHC isoforms at different stages of myoblast differentiation, fixed cultures were blocked with 10% goat serum for 30 minutes at room temperature and then analyzed with the panel of isoform specific, anti-MyHC antibodies using ABC-AP. Cultures were analyzed with all of the Mabs characterized as well as one other slow MyHC-specific Mab (4H3) that was kindly provided by Dr. Jean Leger (INSERM, Montpellier, France, (Bouvagnet *et al.* 1984))

and an affinity-purified, rabbit polyclonal antibody against rat neonatal MyHC (NN6) provided by Dr. Gillian Butler-Browne(Butler-Browne and Whalen, 1984) (U. Biomedicle St. Peres, Paris, France). The specificity of these antibodies is summarized in Table 2.1. In some studies, two commercially available, anti-desmin antibodies (mouse Mab RPN.1101; Amersham, IL, rabbit AB907; Chemicon, CA) were also used to discriminate between myogenic cells and fibroblasts. In all cases, chamber slides were incubated with primary (1°) antibody for 45 minutes at RT. After a 30 minute rinse with several changes of PBS, slides were incubated for 45 minutes at RT in the secondary (2°) antibodies, either biotinylated goat anti-mouse (GAM) IgG (Dimension Lab., Mississauga, Ont) for the mouse monoclonal antibodies or biotinylated horse anti-rabbit (HAR) IgG (Dimensions Lab., Mississauga, Ont) for the rabbit polyclonal antibodies, and rinsed again with PBS and incubated with the avidin-biotinylated alkaline phosphatase complex (ABC-AP) for 1 hr at RT. Following another 30 min rinse in PBS, the slides were incubated for 15 min at RT in substrate buffer (100 mM TRIS, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) containing 3.5 mg NBT, 2 mg BCIP and 2.4 mg levamisol per 10 mls. Slides were washed with PBS, coverslipped with aquamount and stored at 4°C until photographed. Results were photographed on Kodak Tech 2415 film (commercially available) and negatives were all printed under similar darkroom conditions. Colour plates were photographed on Kodak Kodacolor Gold 400 ASA print film (commercially available) and printed commercially.

2.2.6 Fusion Studies

To determine the extent of fusion within myotubes, 47A positive clones were scored for the number of nuclei they contained. 50 myotubes were counted for both embryonic and fetal cultures at two separate time points - 2 and 6 days after plating. The average number of nuclei per myotube (ANNM) was calculated and standard deviations obtained, and from this Student's t - tests were performed. Differences between and within cultures were determined.


b
b'
a
c

2.2.7 Immunofluorescent Co-labelling of Desmin and MyHC Isoforms

Cultures fixed on day 6 were used for immunofluorescent co-labelling in order to examine the co-expression of various MyHC isoforms with each other and with desmin. Plates were blocked with a 10% solution of the appropriate preimmune serum in PBS and 0.1% BSA and the 1° antibodies (diluted in PBS containing 0.1% BSA) were used as previously described. A rabbit anti-desmin polyclonal antibody (Chemicon, CA) was used to detect desmin. Two different sets of 2° fluorescent antibodies were used for the localizations. In the case of anti-desmin/47A, and 47A/NN6 double localizations, the 2° antibodies were a rhodamine-conjugated GAM IgG (Tago, Inc., Burlingame, CA) and fluorescein - conjugated goat anti-rabbit (GAR) IgG (Tago, Inc., Burlingame, CA). For 47A/MY-32 and 8H8/MY-32 double localizations, isotype specific antibodies were used. Fluorescein-conjugated RAM IgG_{2A} (ICN Biomedicals Canada Ltd, Montreal, Que) detected 47A and 8H8, while rhodamine-conjugated sheep anti-mouse (SAM) IgG_1 (The Binding Site, Birmingham, Eng) detected MY-32. Once the 2° antibodies were removed and the cultures washed several times with PBS, they were coverslipped with a mountant containing 50% glycerol and 5% paraphenylene diamine. Hoescht dye 33252 (Sigma Chemical Co. St Louis, MO) was added at a concentration of 0.5% to uniformly label all nuclei within the culture. Fluorescence was photographed on Kodak T-Max 400 film (commercially available) using a Zeiss Axiophot photomicroscope equipped with a filter set 17 for FITC (exciter filter BP485, barrier filter 515-565) and filter set 15 for TRITC (exciter filter BP 546, barrier filter BP 590) using a 40X Neofluar objective. In some cases, double localizations were performed using both the ABC - AP immunohistochemistry and the fluorescent localization.

2.3 Results

2.3.1 Monoclonal Antibody Specificity

A panel of monoclonal antibodies along with one polyclonal antibody was used for all of our studies. The specificity of several of these antibodies has previously been determined and using this information together with an ATPase assay, we were able to verify the specificity of all of the antibodies used in this study (see Figures 2.1 and 2.2 and Table 2.1). The ATP assay identified slow versus fast fibres without discerning between different fast and slow isoforms (Guth and Samaha, 1970). The non-staining fibres represented slow - type fibres while stained fibres represented all the different fast type fibres. IIC fibres also stained and normally would contain both slow and fast IIA MyHC (Brooke and Kaiser, 1970). Characterization of the fast antibodies (Figure 2.1) revealed two antibodies, MY-32 and N1.551, which recognize all of the fast isoforms but none of the slow fibres. Mabs 212F and SC.71 recognized complimentary populations of fast fibres with no overlap of their reactivity. Since published reports have indicated SC.71 to be a IIA MyHC specific Mab, 212F must be recognizing both IIB and IIX fibres. Mab 4A.74, which has a published specificity to IIA MyHC also lightly recognized a second fast isoform. Since these fibres were not recognized by BF.F3, which has a known specificity for IIB MyHC, 4A.74 must be cross-reacting to fast IIX MyHC. Characterization with BF.35 produced a reaction in all fibres except those stained lightly for 4A.74 (a subset of the 212F postitive fibres). This confirmed that BF.35 is recognizing all adult isoform with the exception of IIX MyHC. Characterization of the slow MyHC specific Mabs confirms that they all stained the same population of fibres which did not stain in the alkaline ATPase (Figure 2.2). Monoclonal antibody 47A shows no reaction to any adult tissue, as expected, since its published specificity is for embryonic MyHC only (Sopper *et al.* 1988). The MyHC specificity, isotype and optimal dilution for each Mab is summarized in Table 2.1.

Figure 2.1 Fibre type specificity of fast MyHC specific Mabs in the plantaris muscle of adult Wistar Furth rats. Serial cryostat sections were analyzed using myosin alkaline ATPase histochemistry (A) or ABC-AP immunohistochemistry with Mabs MY-32 (B), BF.F3 (C), 212F (D), SC.71 (E), BF.35 (F), 4A.74 (G), or N1.551 (H). Following myosin ATPase histochemistry, all slow, type I fibres (I) are bleached while fast type II fibres react strongly. Fast fibres can be recognized as type IIA (a), IIB (b) or IIX (x) based upon previously published specificity of each Mab. As expected, MY-32 reacts with all fast fibres (B), BF.F3 reacts with IIB fibres (C) and 212F reacts with both IIB and IIX fibres (D). While both SC.71 (E) and 4A.74 (G) recognize IIA fibres, 4A.74 also shows a consistent, light cross reactivity with IIX fibres. Mab N1.551 reacts with all fast fibres (H) while BF.35 reacts with all adult fibre types except IIX (F). Bar = 170 μm.

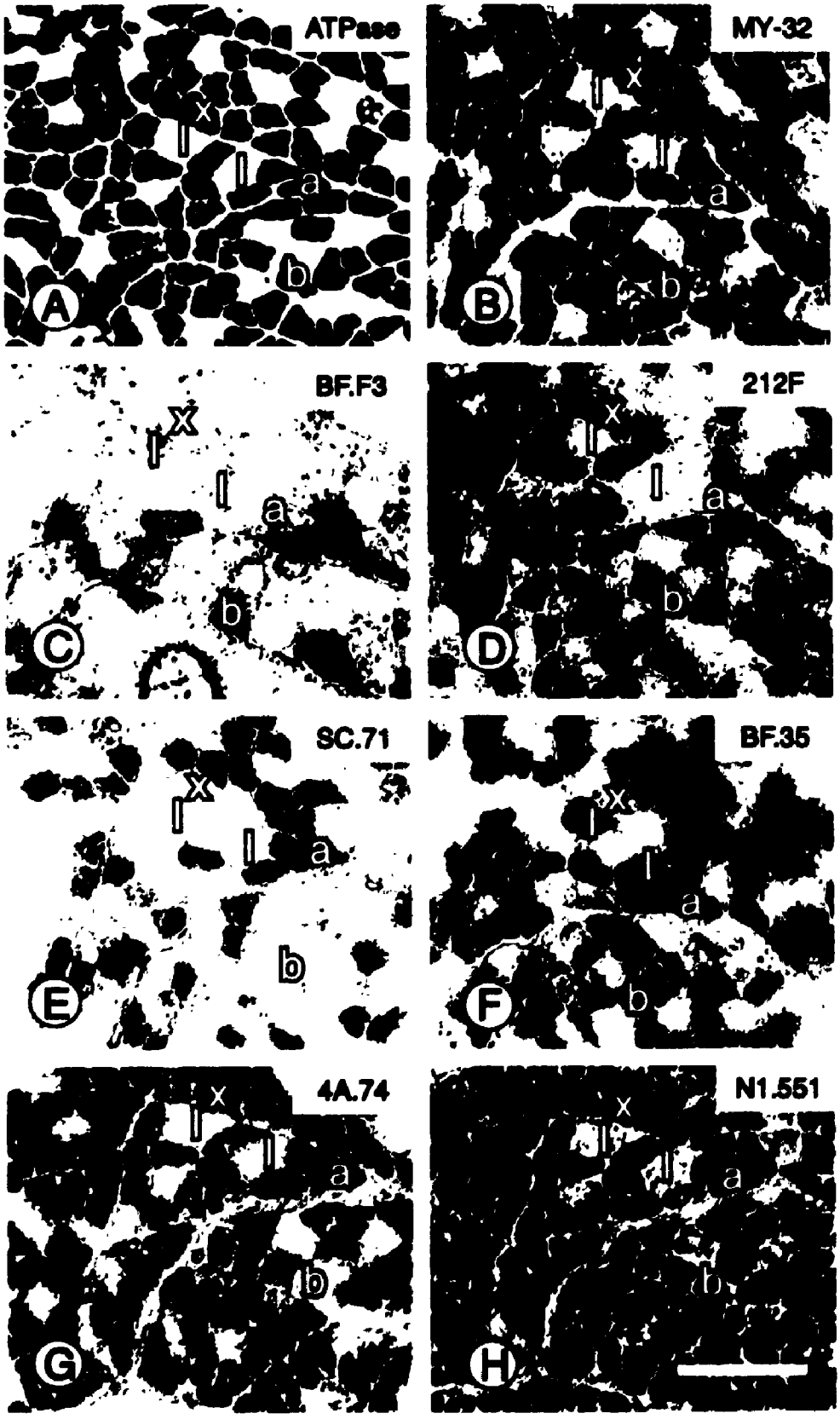
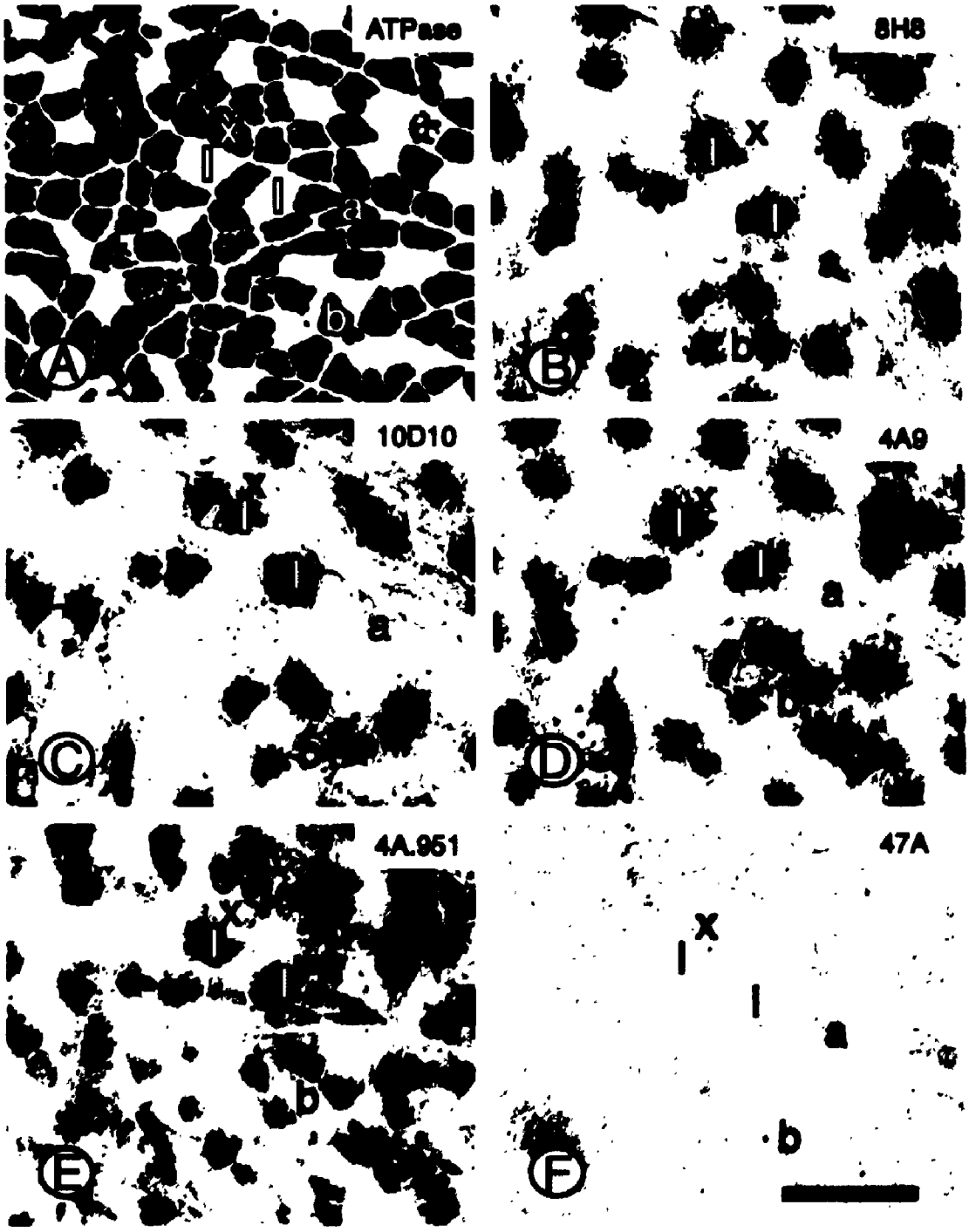

ATPase
MY-32
BF.F3
212F
SC.71
BF.35
4A.74
N1.551
A
B
C
D
E
F
G
H
I
IIx
IIa
IIb

Figure 2.2 Fibre type specificity of slow MyHC specific Mabs in the plantaris muscle of adult Wistar Furth rats. Serial cryostat sections were analyzed using myosin alkaline ATPase histochemistry (A) or ABC-AP immunohistochemistry with Mabs 8H8 (B), 10D10 (C), 4A9 (D), all of which react only to slow fibres, and 4A.951 (E), which shows some cross reaction to type IIA fibres at high concentrations. An embryonic MyHC specific antibody (47A;F) shows the same level of reaction observed in control sections analyzed without primary Mab. Bar = 140 μm.


ATPase
8H8
10D10
4A9
4A.951
47A
A
B
C
D
E
F
a
b
x

2.3.2 Morphological Differentiation of Embryonic and Fetal Myoblasts

Through microscopic analysis, several general statements can be made in regard to the morphological appearance of the two populations of myoblasts. First, it can be seen that there is a large difference in the size potential of the two sets of myotubes. Embryonic myoblasts develop into myocytes (ie. differentiated, mononucleated myotubes) or small myotubes, one to two hundred microns long, while the fetal myoblasts grow to several thousand microns and may be visualized macroscopically (Figure 2.3). Secondly, in cultures of ED14 myoblasts, the increase in the number of myotubes stops after two days in culture, while new myotubes continue to appear for up to 6 days in the fetal cultures (data not shown). Embryonic myotubes are often observed as small sized colonies (possibly clones) while the fetal myotubes cover almost the entire culture dish. Immunohistochemical staining of fetal myotubes demonstrates that they have a high degree of branching, while embryonic myotubes show no branching at all. Finally, it can be seen that the number of nuclei per myotube (NNM) is much higher in cultures of ED20 myoblasts, compared to ED14 myoblasts (Table 2.2).

By scoring 50 myotubes from each culture at days 2 and 6, we obtained an average for the NNM (ANNM). As can be seen in Table 2.2, the ANMM at two days in culture is 1.40 and 7.16 for embryonic and fetal myotubes, respectively. These numbers give a resulting P value of 1.022×10^{-8}, suggesting a significant difference in nuclear number between embryonic and fetal myotubes. After six days *in vitro*, the two populations show an even greater difference in the average number of nuclei. Embryonic myotubes have an ANNM of 1.58 while the fetal myotubes have a much higher ANNM of 33.46 (P value of 2.543×10^{-6}). From the same data, we also determined the statistical significance within the same cultures, but at different time points. The P value for the embryonic myotubes at 2 and 6 days of culture is .1099 (which is not significant) and for fetal myotubes, the value is 5.359×10^{-5} (which is significant). This suggests that embryonic myoblasts complete most of their fusion within 2 days of plating while fetal myoblasts continue to fuse onto

Figure 2.3 Morphological differences between myotubes derived from embryonic (ED14) and fetal (ED20) myoblasts cultured *in vitro*. Cultures of embryonic myoblasts (A,C) or fetal myoblasts (B,D) were fixed at 2 days (A,B) or 6 days (C,D) after secondary plating and reacted with Mab 47A using ABC-AP immunohistochemistry to localize embryonic MyHC. Cells were considered to be myotubes if they reacted positively with Mab 47A. Embryonic myotubes (A,C) do not increase in size or number of nuclei after 3 days *in vitro*. In contrast, fetal myotubes (B,D) continue to increase in size and nuclear number throughout the period of observation. Bar = 270 μm.


B
b'
A
C

Table 2.2 Fusion index of embryonic and fetal myoblasts after two and six days in culture

Number of days in culture	14 ED Cultures				20 ED Cultures			
	Nuclei[a]	ANNM	S.D.	t	Nuceli[a]	ANNM	S.D.	t
2 Days	70	1.40	0.84		358	7.16	6.07	6.684[1]
6 Days	79	1.58	0.64		1673	33.46	44.15	5.122[2]
Comparing timepts. within culture				1.243[4]				4.214[3]

ANNM - average number of nuclei per myotube

1 - $P = 1.022 \times 10^{-8}$ 2 - $P = 2.543 \times 10^{-6}$ 3 - $P = 5.359 \times 10^{-5}$ 4 - $P = 0.1099$

All but t - test 4 is significantly different.

a - total number of nuclei counted in 50 myotubes stained with 47A.

existing myotubes throughout the period of observation.

Embryonic and fetal myotube cultures also differ in their potential to develop extensive sarcomeres and their ability to contract. The embryonic myotubes show very limited, if any, cross-striations. Although there appears to be some form of MyHC arrangement as strands running the length of the myotube, these myotubes show no contractile activity. On the other hand, the fetal myotubes show extensive sarcomeric arrangements with a noticeable cross-striational pattern. These myotubes contract vigorously, starting at day 5, and eventually pull off of the culture dish.

2.3.3 MyHC Expression in Cultures of Embryonic Myoblasts

Monoclonal and polyclonal antibodies against desmin and various MyHCs were used to examine myosin expression in embryonic myotube culture using ABC - AP immunolocalization at days one to eight after secondary plating for both populations of myoblasts (Figure 2.4 and Table 2.3). Embryonic (ED14) myotubes showed a constant reaction with 47A, indicating that they expressed significant levels of embryonic MyHC. However, neither neonatal MyHC nor any adult fast MyHCs could be detected at any of the time points examined. Interestingly, a large number of these myotubes also reacted positively with Mab 4A9 indicating the expression of slow MyHC in many embryonic myotubes. Both embryonic and slow MyHC isoforms could be detected as early as one day after replating and continued to be expressed until at least 10 days in culture. The expression of slow MyHC was corroborated by the binding of Mabs 4H3 and 8H8, which are both specific for slow MyHC (Bouvagnet *et al.* 1984). It was interesting to note that neither 10D10 nor 4A.951, which are also slow specific, reacted with these embryonic myocytes. This indicates that the specificity of these Mabs may be different. To determine the percentage of embryonic myotubes that express slow MyHC, ABC - AP localizations were performed on day 4 cultures (Figure 2.5). Three different Mabs against slow MyHC (Mabs 4A9, 4H3 and 8H8) were used to determine if they were recognizing the same isoform. This localization was followed by immunofluorescent localization

Figure 2.4 Immunohistochemical localization of MyHC expression in embryonic (ED14) myoblasts cultured *in vitro*. Cultures were fixed at 3 days (A,C,G,E) or 6 days (B,D,F,H) after secondary plating and reacted with Mabs against embryonic MyHC (A,B - Mab 47A), slow MHC (C,D - Mab 4A9), adult fast IIB/IIX MHC (E,F - Mab 212F) or a rabbit polyclonal Ab against neonatal MHC (G,H - Ab NN6). Myotubes are small with only a few nuclei per cell (1 - 4) at both 3 days (A,C,E,G) and 6 days (B,D,F,H) in culture. Embryonic MyHC (detected by 47A) is expressed at high levels in all multinucleated myotubes and some mononucleated myocytes at both 3 and 6 days in culture. Slow MyHC is also expressed in many differentiated cells (C,D). Embryonic myoblasts do not accumulate detectable levels of either adult IIB/IIX MHC (E,F) or neonatal MHC (G,H) *in vitro*. Panels reacted with these antibodies have background levels of staining similar to that observed in control wells analyzed with the ABC-AP reagents in the absence of primary antibody (not shown). Bar = 250 μM.

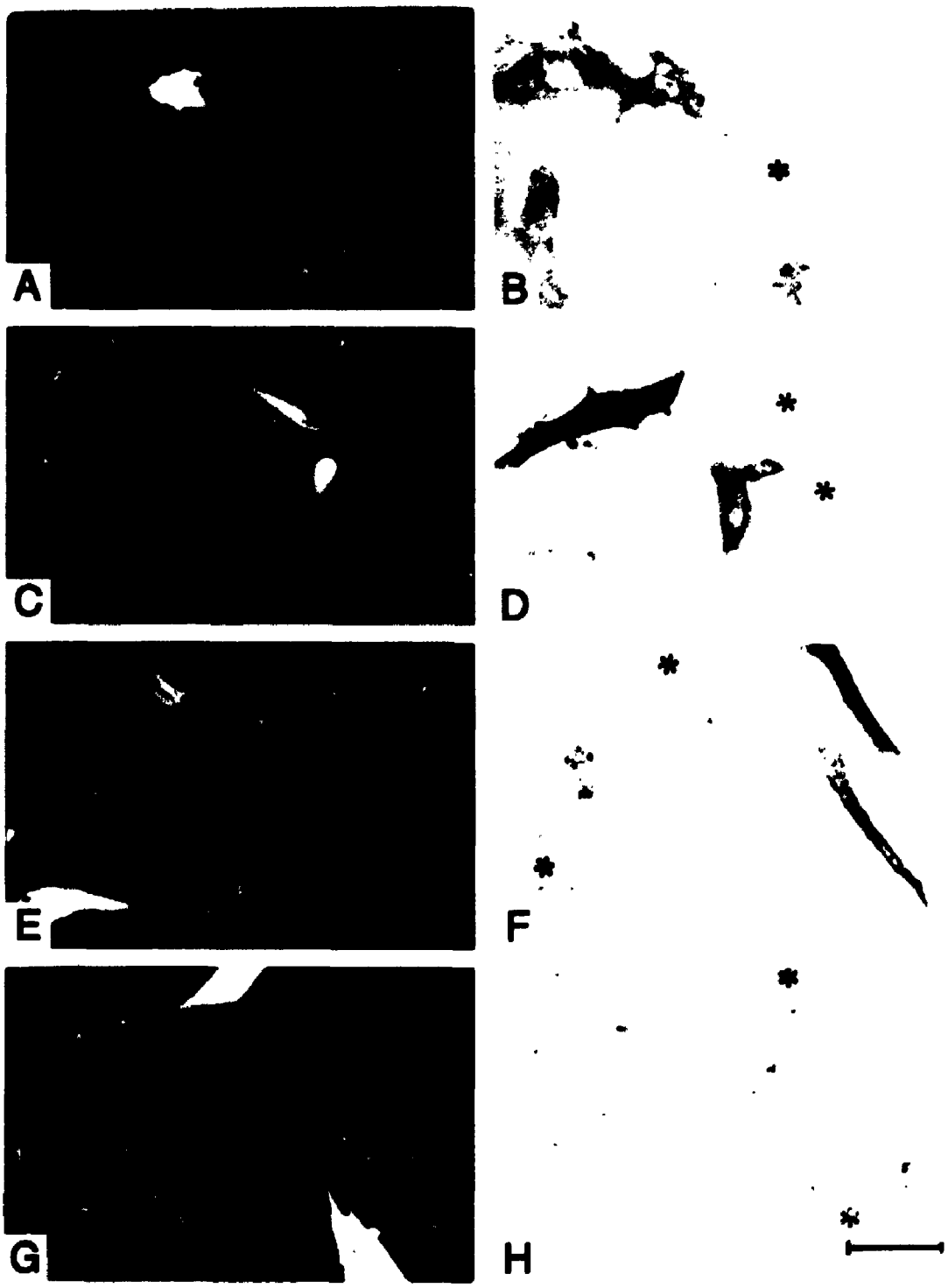

A
B
C
D
E
F
G
H

Table 2.3 Myosin heavy chain expression in embryonic myoblast[a] cultures

Monoclonal Antibody	Days in Culture							
	1	2	3	4	5	6	7	8
47A (embryonic)	+	+	+	+	+	+	+	+
NN6 (neonatal)	-	-	-	-	-	-	-	-
212F (IIB, IIX)	-	-	-	-	-	-	-	-
4A.74 (IIA)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
N1.551 (neonatal, IIA, IIX)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
MY-32 (neonatal, adult fast)	-	-	-	-	-	-	-	-
8H8 (slow)	+	+	+	+	+	+	+	+
4A9 (slow)	+	+	+	+	+	+	+	+
10D10 (slow)	-	-	-	-	-	-	-	-
4A.951 (slow)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
BF-35 (all except embryonic/IIX)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
RPN.1101 (desmin)	+	+	+	+	+	+	+	+

a - myoblasts were obtained from the hind limb buds of rat fetuses at embryonic day 14
n.d. - not determined

Figure 2.5 Comparison of embryonic and slow MyHC expression in embryonic myotubes cultured for 6 days *in vitro*. The right side of the figure (B,D,F,H) illustrates reactions obtained with slow MyHC-specific Mabs 4A9 (B), 8H8 (D) and 4H3 (F) using ABC-AP immunohistochemical localization. Panel H is a negative control for the ABC-AP procedure in which no primary Mab was used. The left side of the figure (A,C,G,E) illustrates the localization of Mab 47A in the same cells (following ABC-AP analysis), using a fluorescein - conjugated, RAM IgG_{2A} secondary antibody. All fields contain myotubes which are embryonic MyHC positive but contain little or no slow MyHC (*). This confirms that embryonic and slow MyHC expression are not co-extensive, and that a small subset of myotubes express embryonic MyHC but not slow MyHC. Myotubes co-expressing embryonic and slow MyHC are negative in the immunofluorescent panels because the ABC - AP reaction product quenches the fluorescence in 47A positive myotubes. Bar = 75 μM.


A
B
C
D
E
F
G
H

with Mab 47A. Counting between 850 and 950 myotubes revealed that the percentage staining for the three slow - specific antibodies all fell within 87 - 94%. Table 2.4 shows that Mab 4A9 labelled 93.3% of the counted myotubes while 4H3 and 8H8 each stained a slightly smaller percentage of the population (87.3% and 89.3%, respectively). This microscopic analysis demonstrates that approximately 90% of the embryonic myocytes and myotubes co-express embryonic and slow MyHCs after 4 days in culture (Table 2.4). Similar results were obtained with cultures fixed at different intervals after plating (data not shown).

2.3.4 MyHC Expression in Cultures of Fetal Myoblasts

Fetal myotubes, on the other hand, exhibited a much different pattern of MyHC expression during differentiation *in vitro* (Figure 2.6 and Table 2.5). Like the embryonic myoblasts, fetal myoblasts started to express embryonic MyHC as soon as they started differentiating. However, the intensity of the immunoreactivity per cell increased with time in culture, concurrent with the increase in nuclear number. In direct contrast to the MyHC profile observed in embryonic cultures, no slow MyHC accumulation, as indicated by Mab 4A9 immunoreactivity, could be detected at any time point. However, upon localization with 8H8, a small population of mononucleated cells were observed at all time points examined indicating that some slow MyHC expression does occur within this population. Fetal myotubes did accumulate other fast MyHC isoforms in addition to embryonic at later time points, however. Immunoreactivity with rabbit antibody NN6 against neonatal MyHC (Butler-Browne and Whalen, 1984) indicated that fetal myotubes started to accumulate neonatal MyHC as early as four days in culture, and accumulated significant amounts of neonatal MyHC over the next 72 hrs. This was confirmed by positive reactions for MY-32, BF.35 and N1.551 (all of which recognize the neonatal isoform), in conjunction with a lack of staining for all adult MyHC specific Mabs. Surprisingly, adult fast IIX MyHC was also expressed in some mature fetal myotubes at 6 days in culture, as indicated by immunoreactivity with Mab 212F but not Mab

Table 2.4 Reactivity of slow monoclonal antibodies in rat primary 14 ED cultures

	4A9	4H3	8H8
Positive myotubes[1]	894	742	823
Total number of myotubes[2]	958	850	922
Percentage of Slow Myotubes[3]	93.3	87.3	89.3

1 - number of myotubes reacting positively in ABC immunolocalization.
2 - number of myotubes reacting positively in ABC or fluoresceine immunolocalizations.
3 - number of myotubes for 1 / number of myotubes for 2

Figure 2.6 Immunohistochemical localization of MyHC expression in fetal (ED20) myoblasts cultured *in vitro*. Cultures were fixed at 3 days (A,D,G,I), 4 days (B,E,H,K) or 6 days (C,F,I,L) after secondary plating and reacted with Mabs against embryonic MyHC (A,B,C - Mab 47A), slow MyHC (D,E,F - Mab 4A9), adult fast IIB/IIX MyHC (G,H,I - Mab 212F) or polyclonal rabbit antibody against neonatal MyHC (J,K,L - Ab NN6). Embryonic MyHC is expressed in all differentiated cells and is a good marker for fetal myotubes at 3,4 and 6 days of culture (A,B,C). In contrast to embryonic myoblasts, fetal myoblasts do not accumulate slow MHC following differentiation *in vitro* (D,E,F). Adult fast IIB/IIX MyHC can only be detected after 6 days in culture in a subset of myotubes (I). Small amounts of neonatal MyHC can be detected in some fetal myotubes at 3 days in culture (J), but this expression is much more clear at 4 and 6 days in culture (K,L). Bar = 190 μm.


a
b
c
d
e
f
g
h
i
j
k
l

Table 2.5 Myosin heavy chain expression in fetal myoblast[a] cultures

Monoclonal Antibody	Days in Culture							
	1	2	3	4	5	6	7	8
47A (embryonic)	+	+	+	+	+	+	+	+
NN6 (neonatal)	-	-	-	+	+	+	+	+
212F (IIB, IIX)	-	-	-	-	-	+	+	+
4A.74 (IIA)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
BF.F3 (IIB)	n.d.	-	-	n.d.	-	n.d.	-	n.d.
SC.71 (IIA)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
N1.551 (neonatal, IIA, IIX)	n.d.	n.d.	-	n.d.	+	n.d.	+	n.d.
MY-32 (neonatal, adult fast)	-	-	-	+	+	+	+	+
8H8 (slow)[b]	n.d.	n.d.	+	+	+	+	+	n.d.
4A9 (slow)	-	-	-	-	-	-	-	+[b]
10D10 (slow)	-	-	-	-	-	-	-	-
4A.951 (slow)	n.d.	n.d.	-	n.d.	-	n.d.	-	n.d.
BF-35 (all except embryonic/IIX)	n.d.	n.d.	n.d.	+	+	+	+	n.d.
RPN.1101 (desmin)	+	+	+	+	+	+	+	+

a - myoblasts were obtained from the hind limb muscles of rat fetuses at embryonic day 20
b - positive myotubes recognized by this antibody were represented by a small percentage of myocytes only.
n.d. - not determined

BF.F3. Neither one of these MyHCs was ever detected in cultures of embryonic myotubes. These observations suggest that, in contrast to embryonic myotubes, fetal myotubes continue to develop over the 6 day period of observation *in vitro*.

2.3.5 Immunofluorescent double localization of Multiple, MyHC Isoforms in Fetal Cultures

To determine the localization of slow MyHC and neonatal / adult fast MyHC, Mabs 8H8 and MY-32 were co-localized in fetal cultures and recognized by isotype-specific, fluorescently-conjugated antibodies (Figure 2.7). Analysis of these cultures at six days after plating confirmed the expression of both isoforms. However, staining with these Mabs appeared to be exclusive with neonatal / adult fast MyHC expression in large multinucleated myotubes and slow MyHC expression confined to mononucleated myocytes. To further examine the phenotypic expression of the various fast MyHCs in the fetal cultures, fluorescently-labelled secondary antibodies were used to colocalize embryonic MyHC with other MyHCs in fetal myoblast cultures fixed 6 days after secondary plating. All myotubes viewed expressed the embryonic MyHC, with a subset of myotubes also expressing the neonatal isoform (Figure 2.8). Neonatal / adult fast MyHC (recognized by MY-32F) was also detected in a subset of differentiated myotubes, but was apparently expressed at lower levels than the embryonic MyHC. These results suggest that only a subset of differentiated fetal myotubes co-expressed neonatal /adult fast MyHC. It is not known whether growing these myotubes longer or under different culture conditions would increase the number of Mab 212F, MY-32 or NN6-positive myotubes. However, since the number of neonatal positive cells increased with time in culture up until day 8, there is no reason why this trend would not continue.

2.3.6 Immunofluorescent double localization of Embryonic MyHC and Desmin

Our immunolocalization experiments with Mab 47A suggested that all fetal myotubes express embryonic MyHC as an early marker of differentiation. To deter-

Figure 2.7 Immunoflurescent co-labelling of slow and neonatal / adult fast MyHC isoforms in fetal myotubes. Fetal myotubes cultured for 6 days were reacted with antibodies specific for neonatal / adult fast MyHC (MY-32; A,C) and embryonic slow MyHC (8H8; B,D). Primary antibody binding was detected with isotype - specific secondary antibodies conjugated with either rhodamine (A,C) or fluoresceine (B,D) using filter combinations specific for each fluorochrome. Low (A,B) magnification reveals that staining for these two isoforms is exclusive with myotubes either being fast or slow, but not both. Higher magnification (C,D) reveals that the 8H8 staining cells are mononucleated, similar to embryonic myocytes, while MY-32 staining myotubes are consistently multinucleated. Bar = 140 µm in A and B, 60 µM in C and D.

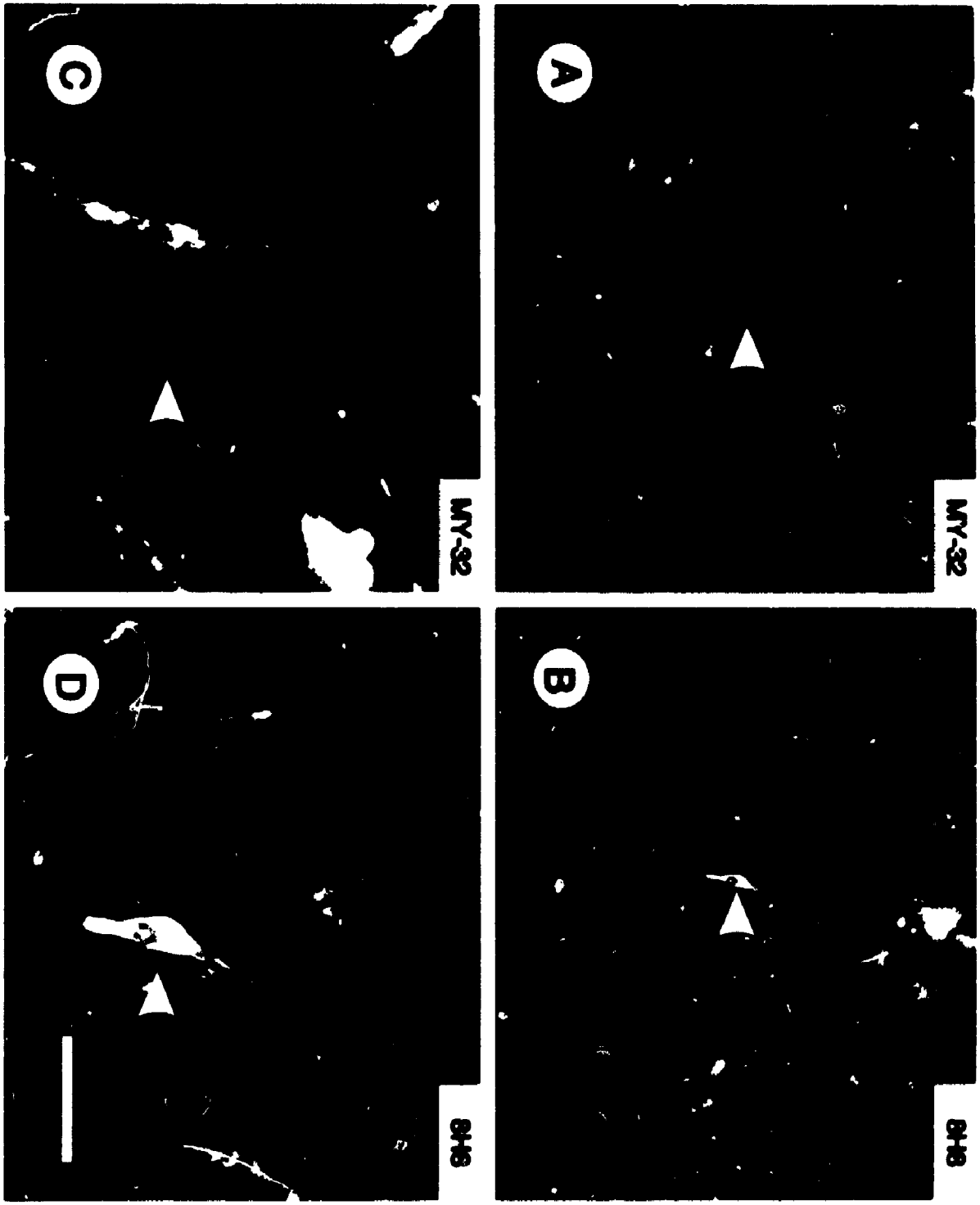
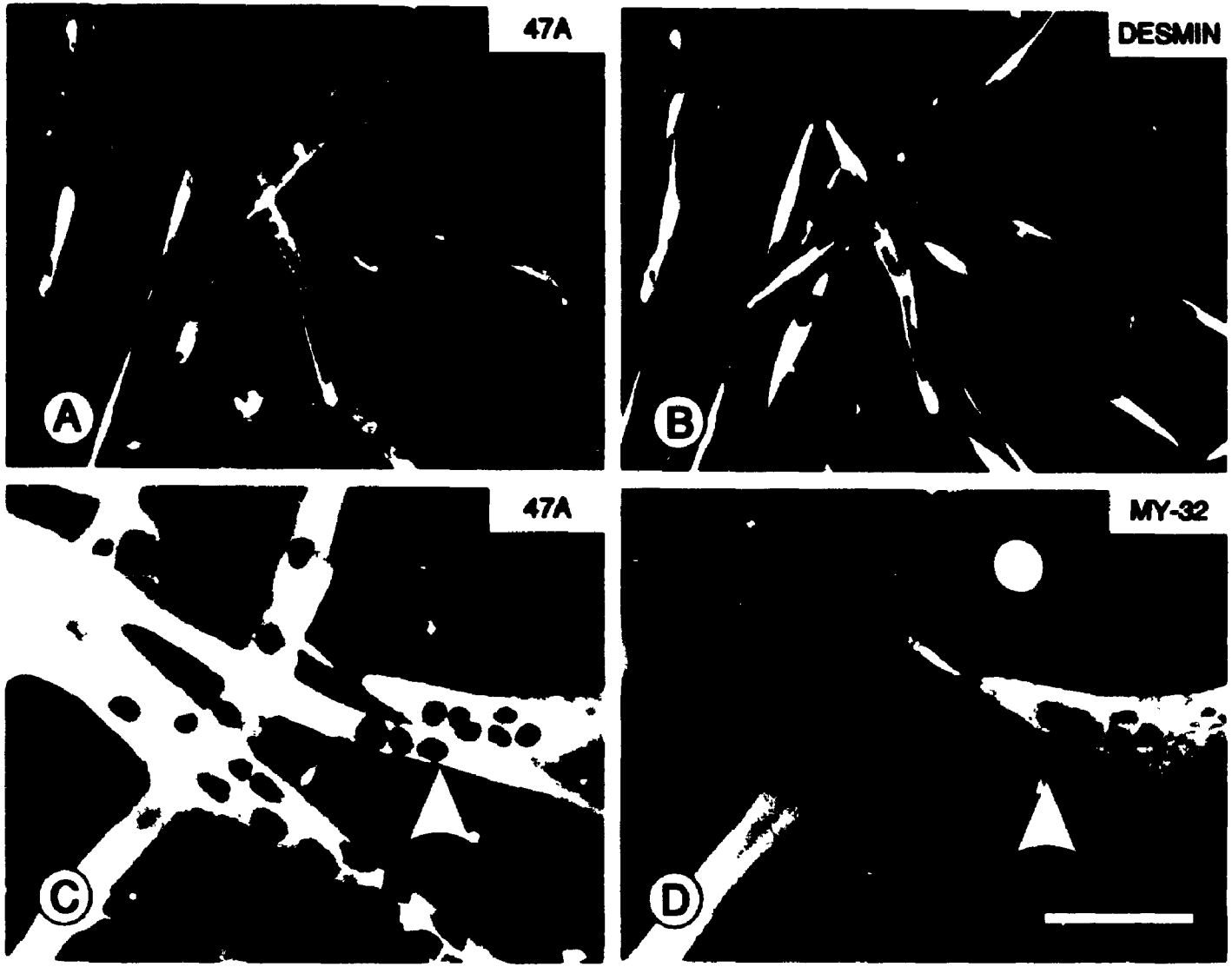

A
MY-32
B
8H3
C
MY-32
D
8H3

Figure 2.8 Immunofluorescent co-labelling of desmin and MyHC isoforms in fetal myotubes. Fetal myotubes cultured for 4 (A,B) or 6 (C,D) days were reacted with antibodies specific for embryonic MyHC (A,C) and desmin (B) or neonatal / adult fast MyHC (D). Primary antibody binding was detected with isotype - specific secondary antibodies conjugated with either fluorescein (A,C) or rhodamine (B,D) using filter combinations specific for each fluorochrome. Localization of desmin and embryonic MyHC using Mabs RPN.1101 and 47A, respectively, demonstrates that all desmin positive myotubes (B) express embryonic MyHC (A). Co-labelling of embryonic MyHC with neonatal / adult fast MyHC using Mabs 47A and MY-32, respectively, demonstrates that while all of the fetal myotubes express embryonic MyHC (C), only a subset of the fetal myotubes coexpress neonatal / adult fast MyHC isoforms (D). Bars = 70 μM.


47A
A
DESMIN
B
47A
C
MY-32
D

mine if there was a sub-population of fetal myotubes which did not accumulate embryonic MyHC, or which lost embryonic MyHC at later stages of differentiation, cultures of fetal myoblasts were fixed after 6 days in culture and analyzed for the co-expression of desmin (using Chemicon rabbit anti-desmin Ab AB907 and an FITC GAM secondary antibody) and embryonic MyHC (using Mab 47A and an IgG_{2A}-specific SAM TRITC conjugate). Using this approach, we observed that all fetal myotubes were immunoreactive for both desmin and embryonic MyHC, suggesting that they are co-extensive (Figure 2.8). This staining pattern was also seen at all other time points examined, which includes days 2, 4 and 8 for both the embryonic and fetal myoblast populations (data not shown). This suggests that all embryonic and fetal myoblasts express embryonic MyHC constitutively following differentiation *in vitro*.

2.4 Discussion

The results demonstrate that myoblasts isolated from hindlimb muscles of ED14 and ED20 rats represent two distinct populations of myoblasts which differ in their ability to fuse and in their expression of myosin heavy chain (MyHC) isoforms *in vitro*. Significantly, the pattern of MyHCs expressed by embryonic myoblasts isolated from ED14 hindlimbs is the same as that reported for rat primary myotubes *in vivo*. Myotubes derived from embryonic myoblasts *in vitro* and primary myotubes generated *in vivo* both co-express embryonic and slow MyHCs but do not express neonatal or adult fast MyHCs (Narusawa *et al.* 1987; Condon *et al.* 1990). This suggests that the embryonic myoblasts which were analyzed in these experiments, and which normally migrate into the rat hindlimb prior to ED15, are the cells responsible for the formation of primary myotubes *in vivo*. Similarly, the pattern of MyHC expression in myotubes formed from ED20 fetal myoblasts *in vitro* is remarkably similar to the pattern observed in secondary myotubes of fetal rat muscle *in vivo* (Butler-Browne and Whalen, 1984; Condon *et al.* 1990), suggesting that fetal myoblasts normally contribute towards the formation of secondary myotubes *in vivo*.

When isolated from the rat hindlimb (at either ED14 or ED20), each myoblast population seems to be predisposed towards forming myotubes which resemble either primary or secondary myotubes, since embryonic and fetal myoblasts have different fusion potentials and generate myotubes with different MyHC compliments when allowed to differentiate *in vitro* under identical culture conditions.

The embryonic myoblasts isolated from ED14 hindlimbs are also similar to the myoblasts that form primary myotubes *in vivo*, in that both populations of myoblasts fuse synchronously over a very short period of time (Harris *et al.* 1989). In cultures of embryonic myoblasts, fusion is almost complete within 48 hours of replating, since the number of myotubes and number of nuclei per myotube is virtually unchanged (ANNM 1.58 vs 1.40) after 2 days in culture. Although these analyses are more difficult to do *in vivo*, myonuclear dating experiments suggest that "primary myotubes form by nearly synchronous fusion of myoblasts with similar birthdates" (Harris *et al.* 1989). This is in marked contrast to secondary myotubes which form in a highly protracted fashion. While primary myotube formation occurs during a brief window in development (ED16 - ED17 in rat lumbricals; (Ross *et al.* 1987)), secondary myotubes continue to form and grow in rats from ED18 until well after birth (Ross *et al.* 1987; Condon *et al.* 1990). Secondary myotubes also express the embryonic, neonatal and adult MyHC isoforms in a predictable, temporal sequence (as implied by their names) during normal development *in vivo* (Condon *et al.* 1990). Thus, the observation that fetal myoblasts fuse and continue to alter MyHC expression patterns over a protracted period *in vitro*, is consistent with the possibility that they are the myoblasts which normally give rise to secondary myotubes *in vivo*.

Although the truncated myotubes formed by embryonic myoblasts *in vitro* are much smaller than the primary myotubes observed *in vivo* (Duxson *et al.* 1989), they are very similar to the myotubes formed *in vitro* by embryonic myoblasts isolated from developing chicks (Miller *et al.* 1985) and mice(Vivarelli *et al.* 1988). This difference in the size of primary myotubes formed *in vivo* and *in vitro* may be due

to limitations of the culture conditions used in these studies. Although the preparations of embryonic myoblasts generally contained higher percentages of contaminating fibroblasts, this does not seem to represent a block to embryonic myoblast fusion, since clones of isolated embryonic myoblasts, free of fibroblasts, also produce small myotubes containing 1 - 4 nuclei (data not shown). In addition, growing embryonic myoblasts at higher densities to increase the probability of cell - cell contact does not significantly increase the degree of fusion (ie. ANNM). Although growing cells on the synthetic extracellular matrix Matrigel increases the proliferation of fetal myoblasts, it does not affect the proliferation or fusion potential of embryonic myoblasts. This is consistent with recent reports from Kaufman's laboratory that embryonic myoblasts do not express H36-α7 Integrin, which is present on fetal myoblasts, and which is necessary for the recognition and binding of laminin in extracellular matrices (Foster *et al.* 1987; Song *et al.* 1992). In spite of these unsuccessful attempts to increase the fusion potential of embryonic myoblasts *in vitro*, it is likely that the poor fusion potential of these cells may be a consequence of inadequate culture conditions, and that under adequate conditions these myoblasts could fuse into large myotubes similar to those observed *in vivo* under different conditions.

Although the embryonic myoblasts differentiate *in vitro* to form a homogeneous population of small myotubes (or myocytes) which all express embryonic MyHC, only about 90% of the differentiated cells co-express slow MyHC. This is similar to the phenotype of mouse embryonic myoblasts allowed to differentiate *in vitro*, where 93-99% of the myotubes co-express slow MyHC with embryonic MyHC (Smith and Miller, 1992). The reason for this apparent heterogeneity within the embryonic cultures is unknown. Since analysis did not reveal large myotubes (~ 4 nuclei) or myotubes which expressed neonatal or adult fast MyHC in these cultures, it is unlikely that the slow MyHC negative cells are derived from fetal myoblasts which migrated into the hindlimbs precociously. Although somitic myoblasts also represent a distinct myogenic lineage (Cossu and

Molinaro, 1987; Cusella-DeAngelis *et al.* 1992), and may have been included in the dissections of ED14 hindlimbs, these myoblasts tend to differentiate much earlier than limb myoblasts *in vivo* and would probably be post-mitotic and/or fused before ED14 and therefore would not contribute significantly to our cultures. At least in the mouse, specialized micro-mass culture conditions, very different from those used in the present study, are required to allow somitic myoblasts to differentiate *in vitro* (Vivarelli and Cossu, 1986). However, since an extensive range in the amount of slow MyHC expressed per embryonic myotube is observed, these Mab 4A9 negative cells most likely represent myotubes which express low levels of slow MyHC which are below the level of detection of our ABC-AP assay.

The observation that fetal myotubes express neonatal MyHC *in vitro* is similar to that previously reported for primary cultures obtained from neonatal rats (Whalen *et al.* 1984). However, it is still not clear whether these neonatal cultures are composed of fetal myoblasts, satellite cells or both. Neonatal MyHC has also been described in differentiated cultures of mouse C2C12 myoblasts (Silberstein *et al.* 1986) and fetal myoblasts obtained from ED12 mice (Smith and Miller, 1992). Although previous authors have suggested that trophic neuronal influences are necessary for the expression of adult fast MyHCs in primary myoblast cultures (Ecob-Prince *et al.* 1986), we have observed significant levels of adult fast MyHC in approximately 10% of the fetal myoblasts cultured without nervous tissue. The sequential expression of embryonic, neonatal and adult fast MyHC mRNAs has also been described in mouse myoblast cell lines (Weydert *et al.* 1987; Cox *et al.* 1991). Since fetal myoblasts express embryonic, neonatal and adult MyHCs sequentially, similar to secondary myotubes *in vivo* (Narusawa *et al.* 1987; Condon *et al.* 1990), these 212F reactive myotubes probably represent the most mature myotubes (ie. the first to differentiate) in our cultures. Presumably, more myotubes would come to express adult fast MyHCs with increased time in culture. While some investigators have suggested that the appearance of neonatal MyHC is induced by contraction of the cultures *in vitro* (Cerny and Bandman, 1986), such a causal relationship was not

observed. Neonatal MyHC is first detected in fetal myotubes prior to the time that they start to contract spontaneously, and cultures of fetal myotubes treated with 6.6 mM $KHCO_3$ to prevent contraction still show accumulations of neonatal MyHC (results not shown).

The ability to obtain pure populations of embryonic and fetal myoblasts from hindlimbs of different ages suggests that, as in birds, rat limbs are infiltrated by two distinct populations of myoblasts which migrate into the limbs at different stages in development (Seed and Hauschka, 1984). The first wave of migrating cells presumably contains embryonic myoblasts which populate the hindlimb at ED14. Since none of the myotubes produced by these cells *in vitro* express neonatal or adult fast MyHC, fetal myoblasts are not present in ED14 hindlimbs and apparently migrate into the hindlimb sometime after ED14. This is different from the situation described in the mouse hindlimb, where Smith and Miller (1992) report neonatal expression in embryonic cultures. Their data consists of 1% positive fibres at ED12 and approximately 25% at ED13, after 6 days in culture. Their results disagree with Vivarelli et al's (1988) finding that there is no neonatal expression in embryonic myoblasts, and their data indicates that there may be a contamination of fetal myoblasts within their embryonic cultures, suggesting that embryonic and fetal myoblasts are both present in ED12 and ED13 mouse hindlimbs. This mixing of myoblast populations in the mouse hindlimb implies that there is much less of a temporal separation between embryonic and fetal myoblast migrations in the mouse, perhaps due to the shorter gestation time of this species. In this study, the presence of very few myocytes (« 1%) which express slow MyHC in cultures of fetal myoblasts demonstrates that the hindlimbs of ED20 rats also contain a relatively pure population of fetal myoblasts. Although this population of slow MyHC positive myocytes may represent lingering embryonic myoblasts, it is also possible that they represent fetal myoblasts that are specifically from the soleus, or even the first wave of satellite cells to slow type muscles. Both of these latter two populations will eventually produce myotubes that are predominantly slow, and culturing of satellite

cells from the soleus has produced slow type myotubes (Düsterhöft and Pette, 1993). Since EM analyses of myoblast fusion in the lumbrical muscles of ED16 rats have shown that most embryonic myoblasts fuse quickly during a 24 hr window and that primary myotube formation is complete by ED17 (Ross *et al.* 1987), the near absence of embryonic myoblasts in ED20 muscle is probably a result of the fact that all of these myoblasts have fused into myotubes prior to ED20. Since the muscle minces were routinely filtered through 50uM nitex cloth during primary culture prior to cell plating, only mononucleated myoblasts and fibroblasts would appear in these cultures. Primary myotubes which had already differentiated would be filtered out and discarded from future analyses.

The demonstration that embryonic and fetal myoblasts from the hindlimb muscles of ED14 and ED20 rats express different phenotypes *in vitro* when cultured under similar conditions, supports the idea that all myoblasts are not created equal, but rather exist as distinct myogenic lineages which may have different developmental origins and fates. The existence of at least 4 distinct myogenic lineages - somitic, embryonic, fetal and satellite cells - has been postulated in mice (Cossu and Molinaro, 1987), and many more have been described in birds (Stockdale and Miller, 1987). Most of these "lineages" have been defined operationally by their behaviour *in vitro*, where different myoblast populations are known to behave differently in terms of their sensitivity to phorbol esters (Cossu *et al.* 1983; Cossu *et al.* 1988), responsiveness to growth factors such as adrenocorticotropin (DeAngelis *et al.* 1992) and TGFβ (Cusella-De Angelis *et al.* 1994), and in their expression of MyHCs (Vivarelli *et al.* 1988; Miller *et al.* 1985) and myogenic regulatory factors such as myogenin (Cusella-DeAngelis *et al.* 1992). At present, the stability and penetrance of the genetic predispositions expressed by different myogenic lineages is unknown. For example, it is unclear whether the embryonic and fetal myoblasts described in this study can fuse with each other and if so, whether one phenotype would dominate. Most likely, the results of such experiments would depend heavily upon the nuclear ratios within each mosaic myotube, similar to heterokaryon

experiments carried out previously with myogenic and non-myogenic cell lines (Pavlath and Blau, 1986). These approaches could be used, however, to start to examine the molecular basis for the establishment and maintenance of distinct myogenic lineages *in vitro*. While many investigators suggest that these different myogenic lineages play important roles in the orderly histogenesis of muscle (Cossu and Molinaro, 1987; Stockdale and Miller, 1987), the stability of these phenotypes *in vivo* is unclear. It is quite possible, for example, that these phenotypes merely represent default genetic programs which are activated artificially *in vitro*, but which are extinguished by dominant environmental influences such as innervation during normal development. To further examine the possibility that embryonic and fetal cell populations represent different myogenic lineages, their fate and ability to fuse with each other will be determined after co-culturing or injection *in vivo*.

CHAPTER 3 - ANALYSIS OF HETEROTYPIC MYOTUBES *IN VITRO*

3.1 Introduction

Since different default patterns were observed in myoblasts derived from ED 14 and ED 20, these two populations appear to have different intrinsic programs of MyHC expression. Although it is possible that these populations represent different myogenic lineages, this can not be confirmed without following their differentiation in various environments both *in vitro* and *in vivo*. The first question to address is whether these populations will fuse with each other and, if they do, what the expressional pattern of the resulting heterotypic myotubes would be.

Skeletal muscles of adult mammals are composed of multinucleated muscle fibres that can be subdivided into different fibre types based upon their speed of contraction (Buller *et al.* 1960) and ability to resist fatigue (Gauthier, 1986). Type I muscle fibres show a slow contractile speed and a high resistance to fatigue, while type II fibres are more susceptible to fatigue yet contract at higher speeds (Pette and Staron, 1990). Type II fibres can also be classified into three subgroups - types IIA, IIX, and IIB fibres - based upon physiological and biochemical criteria (Bär and Pette, 1988; LaFromboise *et al.* 1990). Of these muscle sub-types, IIB fibres contract the fastest and type IIA fibres are the most resistant to fatigue. Type IIX fibres express characteristics similar to both IIA and IIB fibres, and lie somewhere in between the other two fibre types with respect to resistance to fatigue and speed of contraction (Schiaffino *et al.* 1989). Over the last 10 years, myosin heavy chains (MyHCs) characteristic of type I, IIA, IIB and IIX fibre types have been cloned and characterized (Izumo *et al.* 1986; DeNardi *et al.* 1993). Although most adult muscle fibre types express high levels of one particular MyHC isoform (Gorza, 1990), fibres which co-express IIA and IIX MyHC or IIB and IIX MyHC mRNAs have also been identified (DeNardi *et al.* 1993). Each anatomically distinct muscle contains a unique combination of different muscle fibre types, which is intimately related to muscle function (Armstrong and Phelps, 1984). However, the relative importance

of myogenic lineages and environmental influences to the normal development of different muscle fibre types is an area of active debate.

Historically, it has been believed that the phenotype of adult muscle fibres is controlled by the type of innervation (Buller *et al.* 1960; Jolesz and Sreter, 1981; Carraro *et al.* 1986) or pattern of stimulation (Gorza *et al.* 1988; Pette and Düsterhöft, 1992; Termin *et al.* 1989; Pette and Vrbova, 1992) that the muscle fibre receives. For example, fast and slow muscle fibres are innervated by fast or slow motoneurons, respectively (Burke, 1986). At least in adults, cross-innervation of muscle fibres with a foreign motoneuron (Pette and Vrbova, 1985) or electrical stimulation with an inappropriate frequency (Brown *et al.* 1983; Carraro *et al.* 1986; Pette and Düsterhöft, 1992) can transform one fibre type to another. However, the ability of cross-innervation to transform all fibre types equally is still an area of active investigation (Thomas and Ranatunga, 1993; Graybiel, 1990). Recently, studies of muscle development during embryogenesis have demonstrated that the initial differentiation of fast and slow muscle fibre phenotypes does not require innervation (Condon *et al.* 1990), suggesting that different myogenic lineages may play a role in fibre type development in the embryo (Stockdale, 1992). In support of the myogenic lineage model for the maintenance of muscle fibre types, examination of regenerating muscles in the presence or absence of innervation revealed intrinsic control of fast contractile properties in rat soleus muscles damaged with the snake venom notexin (Whalen *et al.* 1990). Combined, these findings suggest that there may be an intrinsic control of muscle phenotype that is independent of, or may work in concert with, environmental influences.

The development of mammalian muscle has been shown to involve the appearance and subsequent fusion of two temporally distinct populations of myoblasts (Ross *et al.* 1987). The first population of myoblasts appears early in the hindlimb development of rat fetuses, first being detected in new hindlimb buds at ED 14 (Condon *et al.* 1990). These myoblasts exhibit a rapid and synchronous pattern of fusion, with the resulting myotubes extending from one end of the muscle bed to

the other by approximately ED 16 (Ross *et al.* 1987). This time point may vary with regard to the distance of the muscle from the axial skeleton, with distal muscles developing later. This first population, which is termed embryonic myoblasts, has essentially finished the fusion process at this time, and continues to grow exclusively through hypertrophy of the myotubes (Harris *et al* 1989). The resulting myotubes, termed primary (1°) myotubes, co-express embryonic and slow MyHC isoforms (Narusawa *et al.* 1987; Condon *et al.* 1990) and will generally develop into adult slow fibres upon maturation (Narusawa *et al.* 1987). At approximately the same time that the embryonic myoblasts finish fusing, a second population of myoblasts appears. Fetal myoblasts are first detected at the motor endplate regions of the existing primary myotubes around ED 17 (Ross *et al.* 1987). These myoblasts will fuse to form secondary (2°) myotubes, which can be distinguished from 1° myotubes since they do not express slow MyHC (Condon *et al.* 1990). Fusion of these myoblasts is somewhat less rigid, with myoblasts fusing onto the ends of the growing myotube. By using the 1° myotubes as a scaffold, the secondary (2°) myotubes gradually extend to the ends of the muscle bed (Kelly and Zacks, 1969; Duxson and Usson, 1989; Harris *et al.* 1989). Growth of these 2° myotubes through fusion continues up to one week postnatally (Wilson *et al.* 1988), and further maturation produces muscle fibres with an adult fast phenotype. Although fetal myoblasts have been shown to initially be within the 1° myotube basement membrane (Harris *et al.* 1989) and electrically coupled to 1° myotubes (Kalderon and Gilula, 1979; Sheard *et al.* 1991), electron microscopic studies suggest that fusion does not occur between the two populations (Duxson *et al.* 1989).

Analysis of these two populations individually in culture has indicated that both populations show *in vitro* characteristics that mirror *in vivo* development of the two myoblast populations (Chapter 2). *In vitro*, embryonic myoblasts differentiate into myocytes and myotubes which co-express embryonic and slow MyHCs, while myotubes derived from fetal myoblasts do not express slow MyHC. This suggests that embryonic myoblasts are predisposed towards forming primary myotubes while

fetal myoblasts are predisposed towards secondary myotubes. As well as maintaining MyHC expression patterns, there is a marked difference in the fusion and maturation of the resulting myotubes in culture, with embryonic myoblasts showing very limited fusion compared to fetal myoblasts. Similar findings have also been documented for other systems, including chick (Stockdale and Miller, 1987) and mouse (Vivarelli *et al.* 1988; Smith and Miller, 1992) models, suggesting that the two populations of myoblasts involved in hindlimb development may represent two distinct myogenic lineages, each establishing a set program of expression prior to fusion. Recent studies in which quail embryonic myoblasts or satellite cell derived muscle precursor cells were injected into embryonic quail supports this argument, since the resulting quail myotubes maintained their *in vitro* phenotype in a foreign environment (DiMario *et al.* 1993; DiMario and Stockdale, 1995). However, neither the effects of innervation upon these fibres, nor the interaction of two different myoblast nuclei within a common environment were addressed by this study, since injected quail myoblasts formed homotypic myotubes which were not innervated.

Studies of heterokaryons, in which fusion occurs between different cell types derived from different species, have shown that nuclear domains can be established (Pavlath *et al.* 1989; Ralston and Hall, 1992). A nuclear domain results from the regionalized production and compartmentalization of transcription (Ralston and Hall, 1992) and translation (Piette *et al.* 1993) products around the nucleus that produced them. Evidence suggests that all mRNA transcripts accumulate immediately adjacent to the nuclei from which they originate (Ralston and Hall, 1992). The characteristics of MyHCs as large, structural proteins, would suggest that they would be localized within the vicinity of transcription (Ralston and Hall, 1989). Studies by Pavlath et al (1989), describing the analysis of muscle heterokaryons resulting from the fusion of human and mouse cell line myoblasts *in vitro*, has shown the localization of species - specific MyHC isoforms around the corresponding nuclei, thereby substantiating this theory. Other studies, involving the localization of acetylcholine receptor proteins, have revealed perinuclear accumulations specifically around

subsynaptic nuclei (Sanes *et al.* 1991). The existence of these nuclear domains may allow one to examine the fate of individual nuclei within a common microenvironment.

In this study, the ability of embryonic and fetal myoblasts to fuse with one another in culture was examined. If embryonic and fetal myoblasts could fuse with each other to form heterotypic myotubes *in vitro*, the next step was to determine if these heterotypic myotubes expressed MyHC isoforms characteristic of embryonic myoblasts, fetal myoblasts or both. Determination of which myoblast population dominated within such heterokaryons could yield important insight into the molecular mechanisms which determine myogenic lineages. To answer these questions, embryonic and fetal myoblasts labelled with various markers were co-cultured and the resulting myotubes analyzed for co-expression of both markers. Our results from this study suggest that embryonic and fetal myoblasts will readily fuse with each other, since heterotypic myotubes containing embryonic and fetal myoblast markers are generated *in vitro*. Interestingly, nuclei within these heterokaryons continue to express MyHC isoforms characteristic of their myogenic lineage in the form of nuclear domains. Nuclei from embryonic myoblasts express slow MyHC while nuclei from fetal myoblasts within the same cytoplasm express perinatal/fast MyHC. These results suggest that MyHC gene expression is tightly regulated in different myogenic lineages and that *cis*-acting elements of MyHC genes, rather than *trans*-acting factors within the cytoplasm, dictate MyHC expression in the different myogenic lineages.

3.2 Materials and Methods

Unless otherwise stated, all chemicals were supplied by BDH Inc., Toronto, Ontario.

3.2.1 Culturing of Embryonic and Fetal Myoblasts

Primary cultures were set up as described previously in Section 2.2.3. Primary plates were set up on 100 mm Falcon tissue culture dishes (Canlab Scientific

Products, Mississauga, Ont), precoated with 100 µg/ml gelatin (Sigma Chemical Co, St Louis, MO) as prescribed by Konigsberg (1979) at a density of 1 x 10^7 cells/ 100 mm dish and incubated overnight in a 37°C incubator containing 5% CO_2. In the case of the fetal cultures, after 24 hrs, cells were selectively trypsinized as described in Section 2.2.3. Final fetal cultures were set up in six-well plates (Canlab Scientific Products, Mississauga, Ont) at various concentrations, depending upon experimental design.

3.2.2 Labelling of Fetal Myoblasts Using Tritiated Thymidine ([^{3}H]Tdr)

Following selective trypsinization, fetal cells were set up at a concentration of 20,000 (low), 50,000 (medium), and 100,000 (high) cells/well on six well dishes. The cells were allowed to settle and attach to the plate for approximately 12 hrs. The complete media (defined in Section 2.2.3) was then replaced with complete media containing 2.5 µCi/ml of [^{3}H]Tdr (ICN Biomedicals Canada Ltd, Montreal, Que). Cells were incubated at 37°C for 47 hrs, and then washed a total of 5 times; CMF-HBSS (Gibco/BRL, Burlington, Ont) two times and complete media 3 times. Two mls of complete media was then added to each well, prior to addition of embryonic myoblasts.

3.2.3 Labelling Embryonic Myoblasts with 5'- Bromodeoxyuridine

5'- Bromodeoxyuridine (BRDU) is a thymidine analogue that is incorporated into replicating cells during the S phase of the cell cycle. To label the cells in our embryonic cultures, pregnant rats were given a daily intraperitoneal injection of 500 µl of 0.9% sterile saline containing 5 mg of BRDU (Sigma Chemical Co, St Louis, MO), for seven days prior to the culture (ie. ED 7 - 13), as suggested by Harris et al (Harris *et al.* 1989). Primary cultures were performed as usual. One embryo from each litter was frozen with melting isopentane and embedded in Tissue Tek OCT (Miles Inc, Elkart, IL) as described in Section 2.2.1.

3.2.4 Labelling Embryonic Myoblasts with PKH26

PKH26 is a lipophilic marker that will insert into the membranes of viable cells (Horan and Slezak, 1989). This marker, which cannot be passed from cell to cell, initially remains on the cell surface, but, with time, will be internalized and label the cytoplasm. Embryonic primary cultures were completely trypsinized with a 1:50 dilution of 20 mg % trypsin (Gibco/BRL, Burlington, Ont) for 10 min. Cells were washed once by centrifugation for 8 min at 800 g followed by resuspension in complete media with serum. This wash was then repeated using Dulbecco's MEM (Gibco/BRL, Burlington, Ont) without serum. After cells were centrifuged a third time, they were resuspended in 1 ml of Diluent C (Sigma Chemical Co, St Louis, MO) according to the manufacturers instructions. At the same time that cells were being prepared for labelling, the PKH26 membrane label (Sigma Chemical Co, St. Louis, MO) was prepared to a concentration of 2.0×10^{-5}. The PKH26 label was added to the cell suspension and cells were incubated at room temperature (RT) for 4 min with the tube being inverted every minute. Following incubation, an equal amount of horse serum (HyClone Labs Inc, Logan, Utah) was added and cells were incubated for one min. An equal volume of complete media was added and cells were centrifuged as usual. Cells were then washed two times with complete media to remove any unbound label, and plated as described below.

3.2.5 Co-culturing of Fetal and Embryonic Myoblasts

Two days after the fetal myoblast cultures had been plated, embryonic myoblasts, labelled with either BRDU or PKH26, were aliquoted onto the same six-well plates at a concentration of 50,000 cells / well. These wells were each fed with 2 mls of complete media, and the media was changed at 2 day intervals. At days 2, 4 and 6 of co-culturing, PKH - cultures were analyzed for expression of PKH26. This analysis included photographing large individual myotubes that showed high levels of PKH26 labelling. Photographs were taken of viable cultures using an inverted microscope, and plates were fixed with 90% methanol at -20°C for 6 min.

Plates were stored at 4°C until MyHC localizations could be performed.

3.2.6 MyHC and BRDU Analysis Using ABC-AP Immunolocalization

To determine the presence of embryonic nuclei within myotubes, and to examine the accumulation of various MyHC isoforms in muscle heterokaryons of myoblast differentiation, fixed cultures were blocked with 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont) for 30 min at 37°C and then analyzed with a panel of isoform specific, anti-MyHC antibodies using the Avidin-Biotin Complex (ABC) method (Hsu *et al.* 1981) in which biotinylated alkaline phosphatase formed part of the complex (ABC-AP). The panel of antibodies against MyHC included monoclonal antibodies used previously to identify embryonic MyHC (47A), slow MyHC (8H8), neonatal and adult fast MyHCs (MY-32) and adult fast IIB/IIX MyHC (212F) in myotubes derived from embryonic and fetal myoblast *in vitro* (Chapter 2). The specificity of these antibodies has already been determined (Sopper *et al.* 1988; Bouvagnet *et al.* 1984) and is summarized in Table 2.1. To detect BRDU, a Mab G3g4 raised against BRDU (NIH Developmental Studies Hybridoma Bank, Iowa City, Iowa) was used, as suggested by George-Weinstein et al (1993). Cultures were first fixed with 95% ethanol for 10 min at -20°C. After several rinses with PBS, the cultures were then pre-treated with 2 N HCl for 30 min at RT. In all cases, plates were incubated with primary (1°) antibody for one hr at RT. After a 30 min rinse with several changes of PBS, slides were incubated for one hr at RT in the secondary (2°) antibody (a biotinylated goat anti-mouse (GAM) IgG (Dimension Labs, Mississauga, Ont) antibody at a dilution of 1:1000) rinsed again with PBS and incubated with the avidin-biotinylated alkaline phosphatase complex (ABC-AP; Dimension Labs, Mississauga, Ont)) for 1 hr at RT. Following another 30 min rinse in PBS, the slides were incubated for 15 min at RT in substrate buffer (100 mM TRIS, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) containing 3.5 mg NBT, 2 mg BCIP (NBT, BCIP; Gibco/BRL, Burlington, Ont) and 2.4 mg levamisol (Sigma Chemical Co, St Louis, MO) per 10 mls.

3.2.7 MyHC Analysis Using Fluorescent Immunolocalization

Wells that had been previously tested for neonatal / adult fast MyHC expression using MY-32 were then analyzed for expression of slow MyHC isoforms. Since the plates had previously been blocked, this step was omitted from the usual immunolocalization. The anti-slow MyHC antibody previously used in the ABC-AP reactions, was used at the same dilution, followed by a 2° fluorescein-conjugated rabbit anti-mouse (RAM) IgG_{2A} (ICN Biomedicals Canada Ltd, Montreal, Que). Once the 2° antibody was removed, the cultures were washed several times with PBS and prepared for autoradiography.

3.2.8 Autoradiography

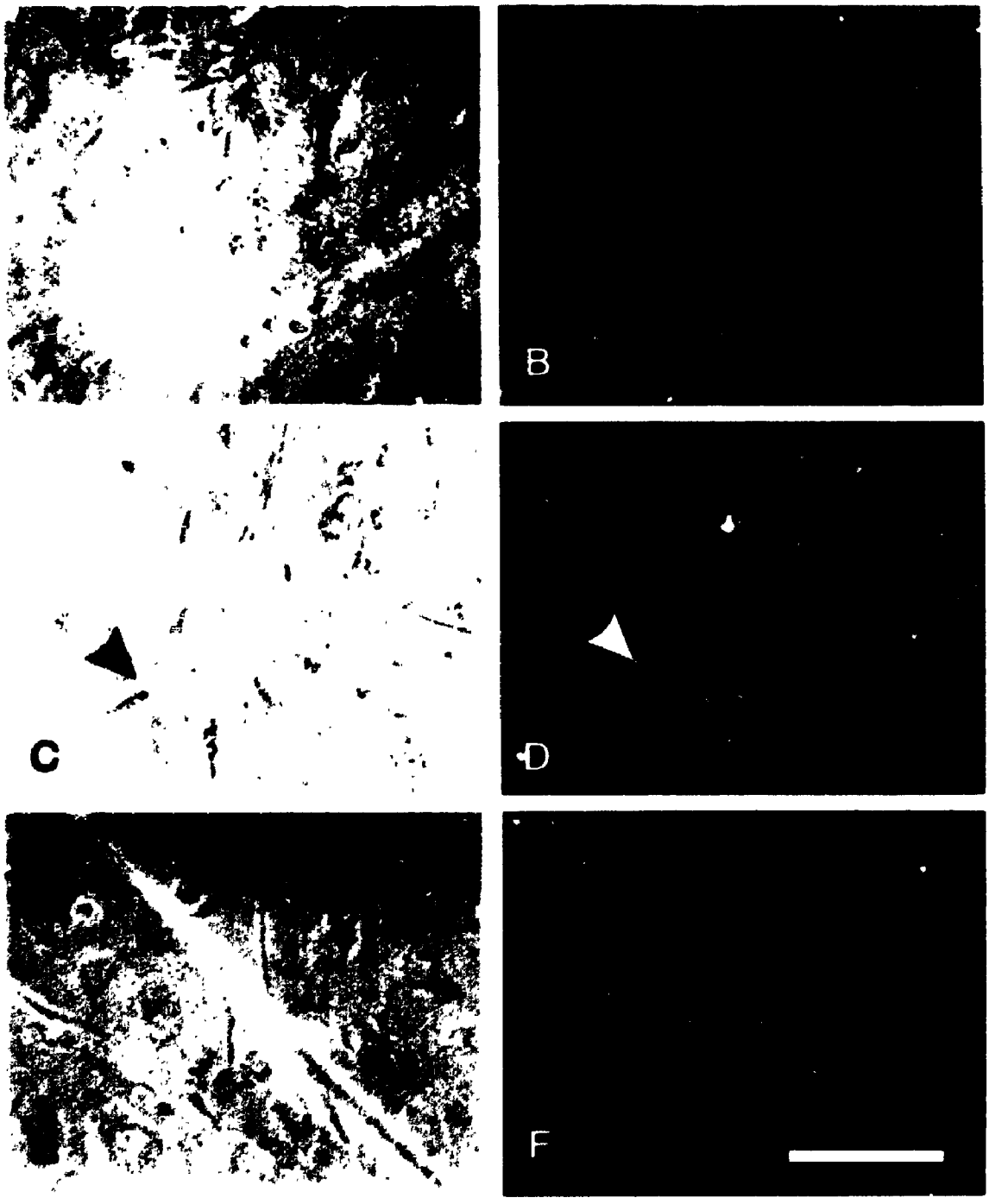
Photographic emulsion (Polysciences Inc, Warrington, Ont) was prewarmed to 40°C and then added to plates at a volume that just coated the surface of the wells. Excess emulsion was quickly drained, and plates were placed on ice until emulsion hardened (~ 10-15 minutes), and then removed until they were dry (45 min). Plates were then placed in black boxes, taped shut, and left at 4°C for six days. After the incubation period, Kodak D19 developer (commercially available) was added to each well and left for four min. The developer was removed, and the reaction was stopped using 0.02% acetic acid for 10 min. This was followed by fixation with 30% sodium thiosulphate for eight min. The plates were then washed with running tap water for 15 min and allowed to dry overnight. This entire protocol was performed under red light only. Plates were coverslipped with a mountant containing 50% glycerol and 5% paraphenylene diamine. Hoescht dye 33252 (Sigma Chemical Co, St Louis, MO) was added at a concentration of 0.5% to uniformly label all nuclei within the culture. Fluorescence was photographed on Kodak T-Max 400 film (commercially available) using a Zeiss Axiophot photomicroscope equipped with a filter set 17 for FITC (exciter filter BP485, barrier filter 515-565) and filter set 15 for TRITC (exciter filter BP 546, barrier filter BP 590) using a 40X Neofluar objective, and negatives were all printed under similar darkroom conditions. Colour plates were photographed on

Fuji 400 ASA colour print film (commercially available) and printed commercially.

3.3 Results

To determine if embryonic and fetal myoblasts can fuse with each other, ED14 and ED20 myoblasts were co-cultured under conditions that allowed for the maximum opportunity for interaction between the two populations. Fetal myoblasts were cultured for two days before the addition of the embryonic myoblasts to allow them to advance to the stage just prior to fusion (Chapter 2). At the time at which embryonic myoblasts were added, no multinucleated myotubes were observed on the culture plates. Cultures were examined immediately after plating the embryonic myoblasts, with two populations of mononucleated cells being observed; one population that labelled intensely with PKH26 that had not yet attached to the plate, and a second population that did not label with PKH26, but was adherent to the plate's surface. Approximately 24 hours after initiating the co-cultures, two populations of adherent, mononucleated cells were observed - one labelled and the other unlabelled - suggesting that fusion had not yet occurred (data not shown). Observing the cultures after 48 hours revealed many multinucleated myotubes, especially on the higher density plates (Figure 3.1). Upon examination of these myotubes under fluorescence, it could be seen that a large proportion of myotubes contained PKH26 label, indicating the contribution of embryonic myoblasts to these myotubes. The PKH26 label appeared to be localized to specific areas of the myotubes, possibly indicating the area in which embryonic myoblasts fused. Examination of embryonic myoblasts alone revealed uniform PKH labelling in all of the cells, which remained mononucleated. Analysis of the cultures four days after co-culture revealed extensive fusion, but PKH26 was no longer regionalized within the myotubes. Instead, the label extended throughout the myotube, at the level of the membrane and within the cytoplasm. This suggests that PKH26 is internalized by the cells that are initially labelled. Five days after co-culture, and seven days after originally plating the fetal myoblasts, most of the larger myotubes had started to con-

Figure 3. 1 Contribution of embryonic myoblasts to multinucleated myotubes following co-culture with fetal myoblasts. Embryonic myoblasts from ED14 embryos were labelled with the lipophylic dye PKH26, and either cultured on their own (A,B) or added to established cultures of mononucleated fetal myoblasts from ED20 embryos and examined after 2 days (A,B,C,D) and 4 days (E,F) of co-culture. Co-cultures were photographed using phase contrast (A,C,E) or fluorescence (B,D,F) optics to demonstrate cell morphology or PKH26 localization, respectively. At 2 days after co-culture, all cells in the single cell culture are positive for PKH26 (A,B). In the co-cultures, large myotubes, as well as mononucleated ED14 cells, contain PKH26 fluorescence (C,D,E,F). In myotubes, PKH26 fluorescence is seen as discrete foci (►) and may represent regions of the myotube contributed by labelled embryonic myoblasts (C,D). Large myotubes containing PKH26 labelling are also observed after 4 days of co-culture (E,F), although PKH26 fluorescence is less discrete and tends to be localized throughout the entire myotube. Scale bar = 220 μm.

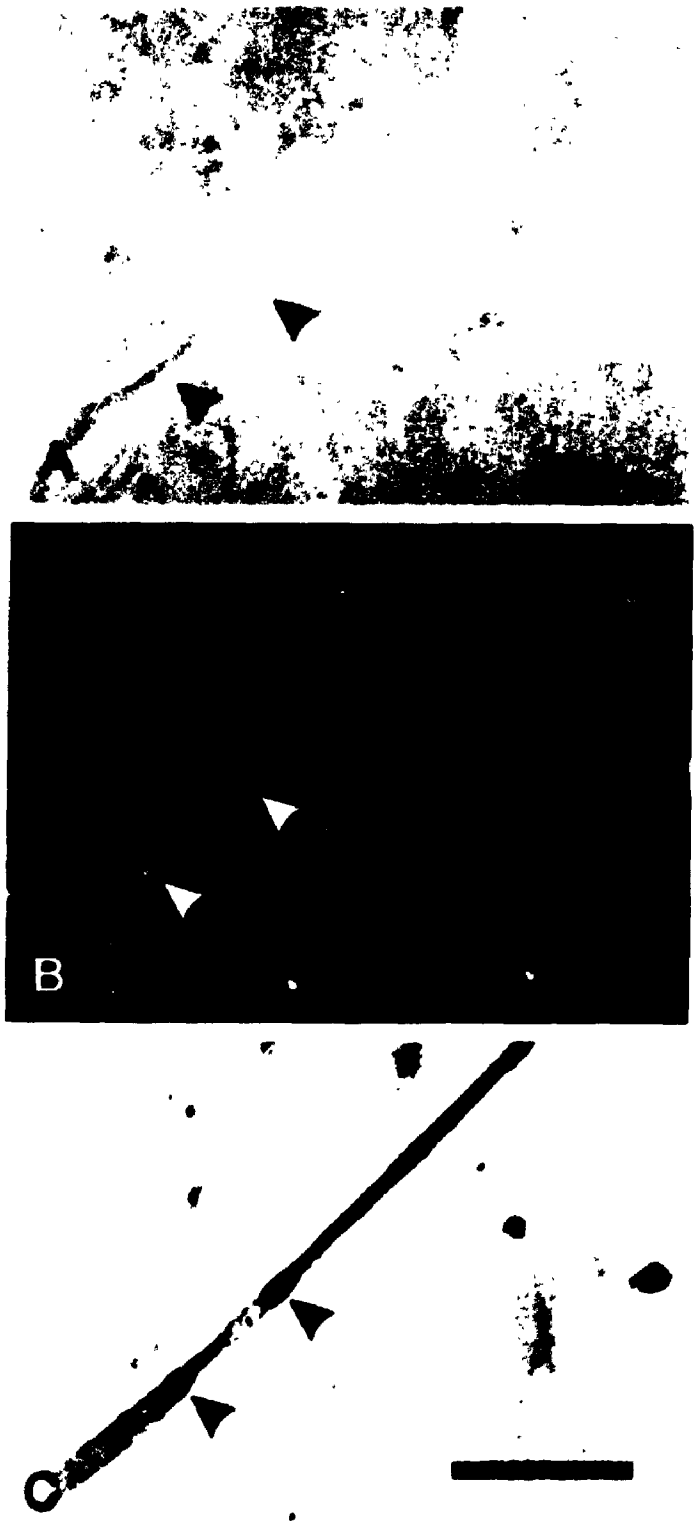

B
C
D
F

tract and lifted off the plates. The majority of labelled cells were mononucleated at this point, and probably represent embryonic myocytes. This pattern of PKH26 labelling was observed for both low and high density cultures. Differentiation of [^{3}H]Tdr - labelled fetal cells in single culture resulted in myotubes with phenotypes similar to those observed for unlabelled cells in Chapter 2 (data not shown).

3.3.1 Embryonic and Fetal Myoblasts Fuse to Form Muscle Heterokaryons by Day Two *In Vitro*

Closer examination of the viable co-cultures revealed more than just localized labelling of PKH26. In some cases, brightly labelled mononucleated cells could be seen lining up along the length of a multinucleated myotube, indicating the presence of embryonic myoblasts in close proximity to established myotubes. To determine if these myotubes represent an increase in the fusion index of embryonic myoblasts, or the formation of myotube heterokaryons from embryonic and fetal myoblasts, it was necessary to analyze the PKH26 - labelled cells for the presence of fetally - derived nuclei. Since the majority of the PKH26 membrane label is lost upon fixation with methanol, individual myotubes showing localized labelling for PKH26 were scored and photographed under standard culture conditions. Cultures were then fixed, analyzed for MyHC expression and then treated for autoradiography. Following autoradiography, scored myotubes from two day co-cultures were evaluated for the presence of [^{3}H]Tdr - labelled nuclei. As seen in Figure 3.2, several myotubes contained both labelled and unlabelled nuclei, with the PKH26 label localized specifically around unlabelled nuclei. Areas around labelled nuclei did not contain the PKH26 marker. This regionalization of the PKH26 appears to initially mark the point of fusion of the embryonic myoblasts onto the myotube. The regional labelling of PKH26 was important to establish the approximate position of the embryonic nuclei since some fetal nuclei remained unlabelled. The existence of PKH26 membrane label and [^{3}H]Tdr - labelled nuclei within a common myotube indicates that fusion between the two populations can occur.

Figure 3. 2 Fusion of [H^3] thymidine-labelled fetal myoblasts with PKH26-labelled embryonic myoblasts to form heterotypic myctubes *in vitro*. Embryonic myoblasts were added to low density cultures of fetal myoblasts and fixed after 2 days of co-culture. Cultures were analyzed using phase contrast optics (A) or PKH26 fluorescence (B) to demonstrate PKH26 labelling in differentiated myotubes. Panel C demonstrates autoradiographic analysis of [H^3] TdR incorporation into nuclei derived from fetal myoblasts, photographed using bright field optics. The presence of [H^3] TdR-labelled nuclei (➤) within PKH26 labelled myotubes demonstrates that embryonic and fetal myoblasts fuse with each other during co-culture to form heterotypic myotubes. Scale bar = 115 μm.


B

To further substantiate these findings, 5'-BRDU - labelled embryonic myoblasts were co-cultured with fetal myoblasts. By injecting pregnant females for seven days prior to establishing a primary culture, it was possible to obtain greater than 95% labelling of embryonic myoblast nuclei (Figure 3.3). Two days after mixing the two myoblast populations, ABC - AP immunolocalization of BRDU using Mab G3g4 revealed myotubes with 5'-BRDU - labelled and unlabelled nuclei. Immunolocalization using the anti-BRDU Mab G3g4 on fetal cultures did not detect any nuclei, while the greater than 95% of the nuclei stained positively in the embryonic cultures . These results confirm the formation of heterotypic myotubes, as previously demonstrated using PKH26 and [^{3}H]Tdr - labelled myoblasts.

3.3.2 Muscle Heterokaryons Maintain Their MyHC Profile in the Form of Nuclear Domains

Prior to treatment of cultures for autoradiography, myotubes were analyzed for MyHC expression using ABC-AP immunolocalization. Figure 3.4 shows a heterotypic myotube containing both the PKH26 membrane marker and [^{3}H]Tdr - labelled nuclei. Localization of slow - type MyHC revealed a regionalized accumulation specifically around a nucleus which did not label for tritiated thymidine. Although the staining lies adjacent to a labelled nucleus, it does not extend beyond, leaving more than half the myotube devoid of slow - type MyHC. This staining specifically recognized the area where the PKH26 label localizes, indicating the presence of a nucleus derived from an embryonic myoblast in the immediate vicinity. In sharp contrast, a nearby myocyte, which stained for PKH26 and contained no [^{3}H]Tdr - labelled nuclei, demonstrated the characteristic embryonic myocyte appearance in culture. It can be seen that staining for slow MyHC extended throughout the entire myotube.

Colocalization of both slow and neonatal/adult fast MyHCs revealed the occurrence of two separate domains within the same myotube. Figure 3.5 demonstrates a myotube that contains both labelled, and unlabelled nuclei. Examina-

Figure 3.3 Immunohistochemical localization of BRDU in nuclei of embryonic myob[illegible]s cultured alone (A,C) or co-cultured with fetal myoblasts for 2 days (B). Embryos used for the isolation of embryonic myoblasts were labelled with BRDU *in utero* between ED7 and ED14 to allow incorporation of BRDU into dividing myoblasts. Comparison of embryonic myoblast cultures following ABC-AP immunolocalization of BRDU using bright field (A) and phase contrast (C) optics reveals that almost all embryonic myoblasts have incorporated detectable levels of BRDU. BRDU localization after 2 days of co-culture with unlabelled fetal myoblasts reveals BRDU immunoreactive nuclei (white arrow head) contributed by embryonic myoblasts within a myotube containing predominantly unlabelled (►) nuclei (B). Pure cultures of fetal myoblasts allowed to differentiate and analyzed for BRDU incorporation using ABC-AP immunohistochemistry do not react with G3g4 antibody (D). These results confirm the presence of nuclei derived from embryonic and fetal myoblasts within a common cytoplasm two days after co-culture. Scale bar = 90 μm.

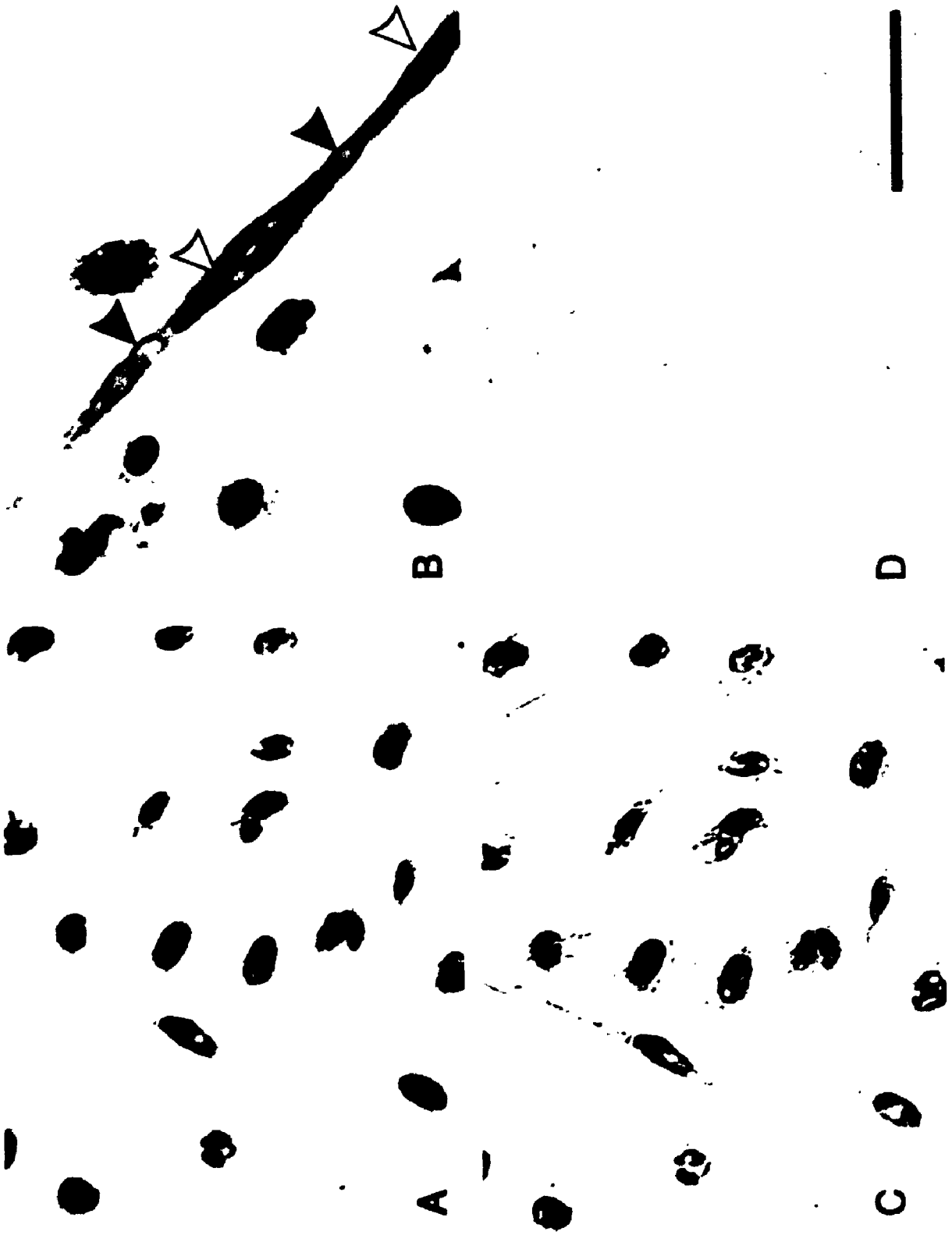

B
D
A
C

Figure 3.4 Regionalized expression of slow MyHC in heterotypic myotubes resulting from the fusion of embryonic and fetal myoblasts. PKH26-labelled embryonic myoblasts and [H^3] thymidine-labelled fetal myoblasts were co-cultured for 2 days, fixed and analyzed using (A) phase contrast optics, (B) PKH26 fluorescence and (C) ABC-AP localization of slow MyHC followed by autoradiography. A heterotypic myotube (➤) contains both PKH26 (B) and [H^3] thymidine (C; labelled nucleus indicated by open arrow) label while a homotypic myotube derived exclusively from embryonic myoblasts (→) contains only PKH26 label (compare B and C). ABC-AP immunolocalization using Mab 8H8 reveals accumulation of slow MyHC in both myotubes (C). The homotypic myotube shows slow MyHC immunoreactivity typical of embryonic myoblasts after differentiation in culture. However, slow MyHC in the heterotypic myotube is regionalized around an unlabelled nuclei derived from embryonic myoblasts. This suggests that nuclei derived from embryonic myoblasts express slow MyHC even when combined with fetal myoblast nuclei within a common cytoplasm. Scale bar = 130 µm.

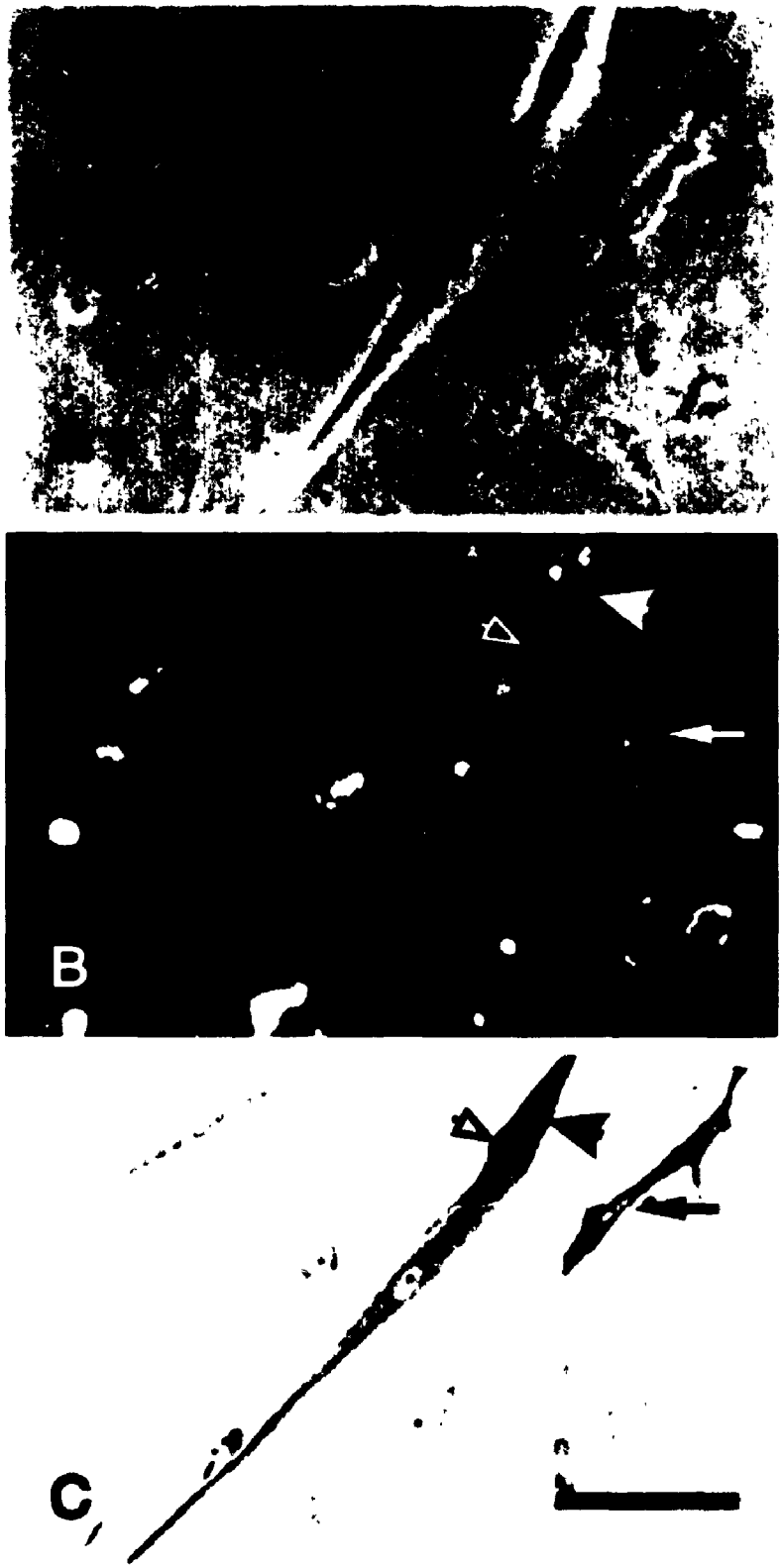
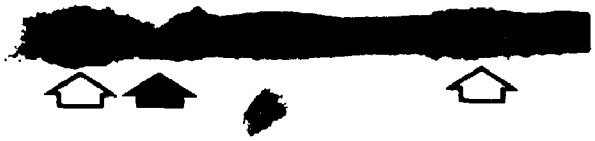
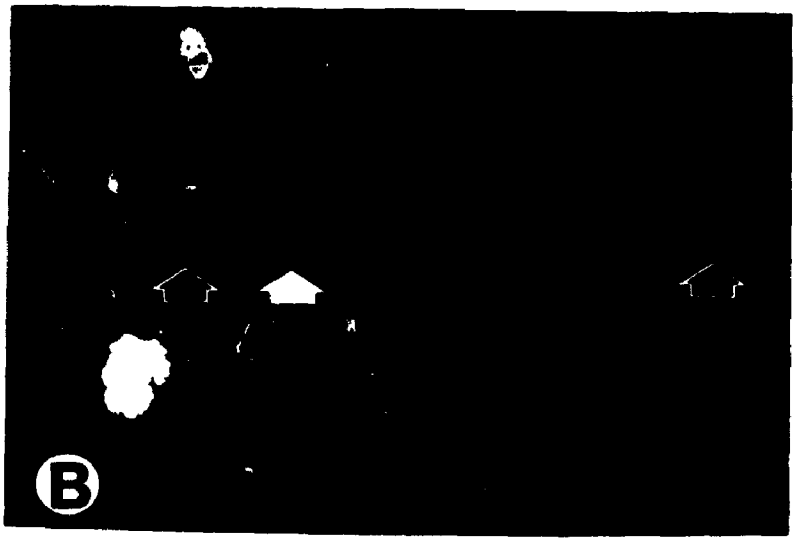
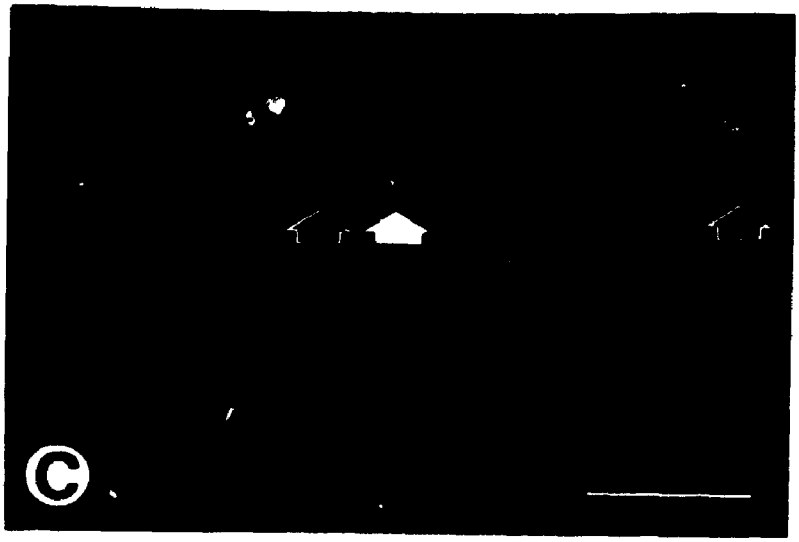

B
C

Figure 3.5 Heterotypic myotubes derived from the fusion of embryonic and fetal myoblasts show regionalized expression of neonatal/fast and slow MyHC isoforms in the form of nuclear domains two days after co-culture. A typical heterotypic myotube analyzed using autoradiography / neonatal/adult fast ABC-AP immunolocalization (MY-32;A), immunofluorescence localization of slow MyHC using an FITC-labelled secondary antibody (8H8;B), and PKH26 fluorescence (C). When PKH26-labelled embryonic myoblasts were co-cultured with [H^3] thymidine-labelled fetal myoblasts for two days, heterotypic myotubes with both [H^3] thymidine-labelled nuclei (○) and PKH26 fluorescence were observed (compare A and C). ABC-AP immunohistochemical localization of fast MyHC (A) reveals co-localization of neonatal/fast MyHC with one of the labelled nuclei derived from fetal myoblasts. In contrast, unlabelled nuclei (●) associated with PKH26 labelling (C) accumulate slow MyHC as a nuclear domain (B). Bar represents 100 μm in panels A and 45 μm in panels B,C.



A

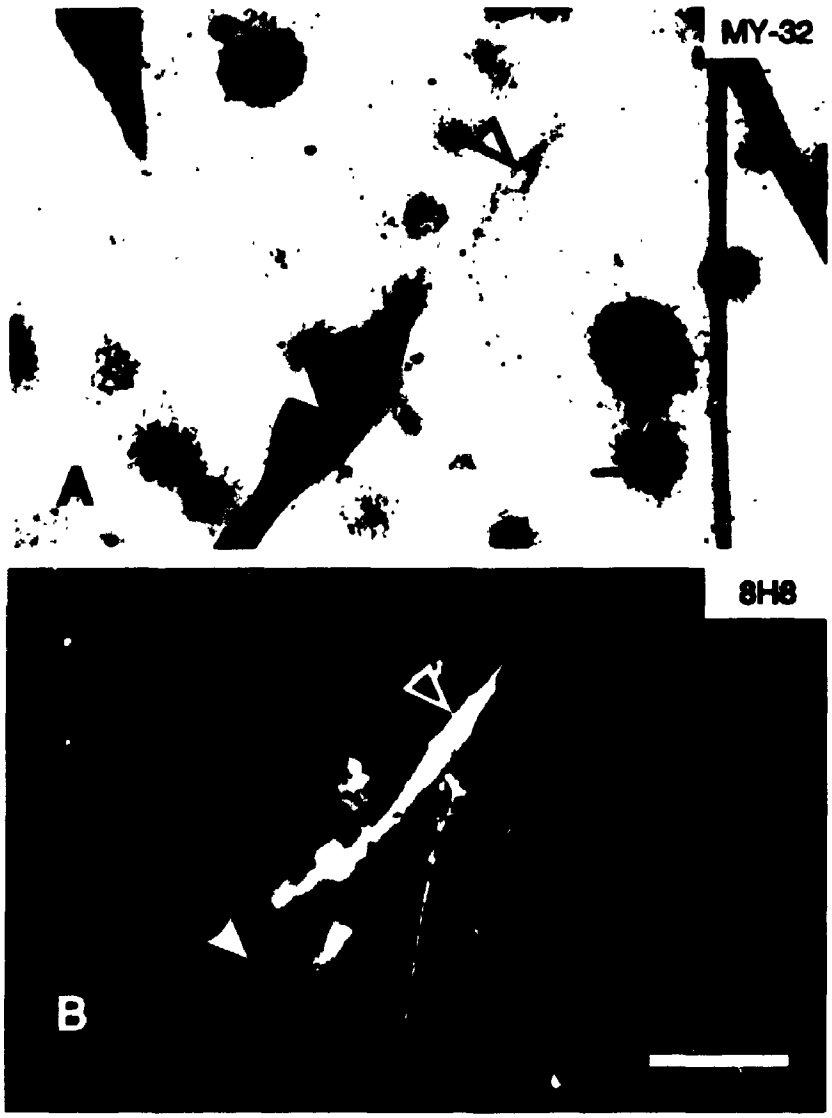




tion with a neonatal/adult fast MyHC Mab revealed that this isoform specifically localizes around a fetally - derived nucleus. The other labelled nucleus within this myotube did not show a nuclear domain of this nature, suggesting that it has not developed as far along the differentiation pathway as the other fetally-derived nucleus. Previous analysis of pure cultures of fetal myoblasts demonstrated the presence of myotubes expressing embryonic and neonatal/fast or exclusively embryonic MyHCs at similar times in culture (Chapter 2). Between these two nuclei, is a third, unlabelled nucleus. Analysis of the myotube using the slow MyHC antibody (8H8) revealed a region of slow MyHC accumulation around the unlabelled nucleus. Upon further examination, it could be seen that the myotube also showed the remnants of PKH26 labelling, specifically around the nucleus of interest.

Further examination of the cultures revealed other myotubes co-expressing both slow and neonatal/adult fast isoforms (Figure 3.6). Analysis of these nuclear domains revealed a high degree of variability in their length. By measuring the extent of the domains around embryonic nuclei, the average size of these domains was estimated as approximately 50 - 100 μm in length. Unfortunately, measurement of fetally - derived nuclear domains was not possible. The domains examined (and shown here), did not appear to show a high amount of overlap in their expression. The best example illustrating this kind of segregration was seen in Figure 3.4, in which a very limited nuclear domain of slow MyHC was observed, and where the nucleus contributed by an embryonic myoblast is surrounded at close proximity by fetally - derived nuclei. This suggests that the lineage-specific MyHC isoforms do not assemble into the same thick filaments.

Figure 3.6 Heterotypic myotubes derived from the fusion of embryonic and fetal myoblasts show regionalized expression of neonatal/fast and slow MyHC isoforms in the form of nuclear domains two days after co-culture. A typical heterotypic myotube analyzed using autoradiography / neonatal/adult fast ABC-AP immunolocalization (MY-32;A) and immunofluorescence localization of slow MyHC using an FITC-labelled secondary antibody (8H8;B). Once again, the [H^3] thymidine-labelled nucleus (•) is surrounded by neonatal/adult fast MyHC (A) while the unlabelled nucleus (○) (presumably derived from ED14 cells) is surrounded by slow MyHC (B). Bar = 100 μm.


MY-32
A
8H8
B

3.4 Discussion

The results clearly demonstrated that embryonic and fetal myoblasts will fuse with each other *in vitro* to form heterotypic myotubes, since markers for each myoblast population can be found within individual myotubes. However, this should not be taken as proof that these myoblasts represent a common lineage, since fusion of genetically distinct myoblasts from different species has previously been reported (Yaffe and Feldman, 1965; Pavlath *et al.* 1989; Noursadeghi *et al.* 1993). What this result does suggest is that there are mechanisms at work in the developing embryo to prevent the formation of heterotypic myotubes *in vivo*, since careful EM analysis of muscle development *in vivo* has shown that fetal myoblasts do not normally fuse with 1° myotubes (Duxson *et al.* 1989; Harris *et al.* 1989). While regional differences in extracellular matrix could provide such a barrier to fusion, temporal differences in myoblast membrane remodelling during differentiation could also provide a barrier to fusion *in vivo* which would be absent from our *in vitro* experiments.

The fact that fusion does not occur between primary myotubes and fetal myoblasts *in vivo* suggests that either a barrier has been established within the fibres, or that a surface signal that was present in the embryonic myoblasts is down-regulated after fusion. The idea that fusion, or differentiation, is important for this down-regulation may be supported by the pattern of fusion that these myoblasts exhibit both *in vivo* and *in vitro*. *In vivo*, the cells line up along the entire length of the fibre and fuse in synchrony (Ross *et al.* 1987), while *in vitro* the embryonic myoblasts usually differentiate before they have a chance to migrate and line up with one another (Chapter 2). This manner of fusion suggests that there is a small window of opportunity for fusion to occur. A possible model for fusion might include a surface marker that is temporally expressed allowing the myoblasts to communicate and prepare themselves for fusion. Possible membrane proteins that might be involved in this process are gap junction protiens (Sheard *et al.* 1991; Balogh *et al.* 1993), specific isoforms of NCAMs (Moore and Walsh, 1985;

Figarella-Branger *et al.* 1992; Peck and Walsh, 1993), cadherins (Mege *et al.* 1992) or integrins (George-Weinstein *et al.* 1993), all of which show temporal expression in myotube development, but are absent in mature muscle fibres. The proteins involved in this process should be maintained for longer periods of time on 2° myotubes, allowing fusion of myoblasts onto the myotubes, similar to what is observed *in vivo* and *in vitro*.

Alternatively, the difficulty of following fusion using morphological criteria alone, may have caused previous investigators to overlook normal fusion between embryonic and fetal myoblast populations. Recently, Evans et al (1994) used *in vivo* cell labelling with retroviral vectors to demonstrate that single clones of cells fuse to both primary and secondary fibres during development. However, they did not specify the manner in which the cells were being incorporated. Although the clones could be contributing to normal developing fibres, it is equally likely that they may be contributing to the regeneration of damaged fibres, which can exhibit focal necrosis (Papadimitriou *et al.* 1990).

Upon analysis of the heterokaryons using MyHCs as a marker for phenotype, it could be seen that individual nuclei within a common cytoplasm were expressing different isoforms. As would be expected from two different myogenic lineages, the nuclei were expressing the isoforms characteristic of the populations they were derived from. It is interesting that such regional expression should take place, given that heterokaryons derived from myoblasts and hepatocytes exhibit organelle arrangements and proteins characteristic of muscle, rather than maintaining both muscle and liver phenotypes (Miller *et al.* 1988). However, this transition within the hepatocyte nuclei occurred at later time points in culture and may represent the appearance of a more developed heterokaryon phenotype.

The fact that MyHC can be expressed in the form of nuclear domains has previously been documented *in vitro* (Pavlath *et al.* 1989). These reports have placed the length of such domains between 50 and 100μm, which correlates with the distances observed here for embryonically - derived domains. In the case of the

neonatal/adult fast domains, it is hard to give a reasonable estimate of their extent due to three factors. First, the fetal nuclei occur at a much higher rate within the myotube, and there may be overlapping domains that would confuse the results. Secondly, not all fetally - derived nuclei express the neonatal/adult fast isoforms that define their domain. Therefore, false negatives would improperly decrease the measurement of the domain. Finally, detection using ABC-AP often obscures the nuclei of interest, making it difficult to get an accurate measurement. However, the fact that nuclear domains are seen for both populations within the same myotube indicates that the influences of one population on another within the myotube do not override any pre-programming that has occurred within the myoblasts prior to fusion. This pre-programming of the myoblasts suggests that the two populations are distinct and possibly represent two separate myogenic lineages, one expressing a slow phenotype that appears early in development, and a second population that appears later and expresses a fast phenotype.

There are several possible models that would explain the establishment of different myogenic lineages. Recent evidence from Hughes et al (1993) indicates that relative levels of different myogenic factors are correlated with fast and slow fibre types. It could be that such quantitative differences of these factors help in establishing the pre-programming occurring within the muscle precursor cells. It is also possible that these two populations are distinct due to expressional differences of "muscle fibre - typing" genes. Although there have been no such genes yet identified, such genes have been shown to exist for the differentiation into both mesoderm and muscle specific cell types. For example, the MHox gene has been shown to convert cells of endodermal origin into cells of mesodermal origin (Cserjesi *et al.* 1992), while the myogenic regulatory factor genes convert many different cell types to skeletal muscle (Weintraub *et al.* 1989). However, both of these mechanisms describe regulation through *trans*-acting factors, which is unlikely to account for such regionalized expression of MyHC in culture. Soluble factors which are necessary to cause transactivation of genes will diffuse throughout the length of

the myotube, similar to ß-galactosidase (Ralston and Hall, 1989). Since there are defined nuclear domains established in muscle hybrids, it is more likely that *cis*-acting elements are controlling MyHC phenotype. This possibility would involve the regulation of specific enhancer or promoter regions in a subset of the MyHC genes. Cloning of MyHC promoter regions has revealed the presence of specific *cis*-acting elements (Yu and Nadal-Ginard, 1989), and A+T-rich sequences which can bind specific myocyte enhancer factors (MEFs) (Lakich and Whalen, 1994). The accessability of these sequences in only a subset of the MyHC gene promoters may implicate these factors in myogenic lineage programming. Methylation of specific gene enhancers or promoters could account for the short term repression of gene expression (Voytik *et al.* 1994). Preferential hypomethylation of MyHC genes at these regions in only one of the populations could lead to the differential expression reported here (Donoghue and Sanes, 1994). If this is the case, culturing the myoblasts in the presence of 5-azacytidine should eliminate the expressional differences found between the two populations.

Finally, it is possible that the regionalized expression of MyHCs is only transient, and that nuclear domains may not be seen in heterokaryons maintained for longer periods of time. Unfortunately, the culture conditions used in this study do not allow for the long term culture of myotubes. Studies by Hughes and Blau (1992) have suggested that myoblasts are reprogrammed upon fusion *in vivo*, indicating the effect of *trans*-acting factors in this system. However, the myoblasts that they injected showed no phenotypic limitations in MyHC expression *in vitro*, unlike the populations described here. To correctly address this question, injection of both embryonic and fetal myoblasts into different environments *in vivo* needs to be performed. Using such an injection regime should allow one to follow myoblasts for at least four weeks *in vivo* to examine the long term stability of nuclear domains in a foreign environment

CHAPTER 4 - INJECTION OF PRIMARY MYOBLAST POPULATIONS INTO THE CAUDATE-PUTAMEN OF ADULT RATS

4.1 Introduction

The observation that embryonic and fetal cells exhibit vastly different phenotypes in culture suggests that these two populations represent different myogenic lineages, with their final phenotype governed in part by intrinsic differences. However, it is possible that the intrinsic differences which establish a particular phenotype *in vitro* may be modulated by environmental influences present *in vivo*. To address this possibility, it is necessary to examine the developmental potential of each cell population following transfer into *in vivo* environments which strongly challenge the internal programs that have been established.

Although the phenotypes of embryonic and fetal myoblasts differ *in vitro* for rats (Chapter 2), mice (Smith and Miller, 1992; Vivarelli *et al.* 1988) and quail (Stockdale, 1992), it is possible that extrinsic factors may override these intrinsic programs. The absence of many of the adult isoforms, as well as the continued expression of developmental MyHC isoforms throughout the length of the culture, supports the idea that full maturation of myotubes is not obtained *in vitro*. Co-culturing of muscle with spinal cord has provided evidence that trophic factors exist which allow adult isoforms to be up-regulated (Ecob-Prince *et al.* 1986). However, studies that characterized the MyHC profiles of various mouse cell lines indicated that adult MyHC isoforms can appear without external factors being present (MacIntyre, 1995). To address the role of environmental influences on muscle phenotypes, researchers have now turned to examining the fate of myoblast populations *in vivo* using myoblast transplantation (Blau and Hughes, 1990; Blau and Hughes, 1990; Anderson *et al.* 1991).

Myoblast transplantation, which involves the injection of myogenic precursor cells into muscle, has been suggested as a possible therapy for most inherited

myopathies (reviewed by Karpati, 1990; Karpati *et al.* 1993; Pagel and Morgan, 1995; Partridge, 1991). Although clinical trials have been disappointing, the technique has been useful in addressing basic scientific questions on muscle development. The standard procedure involves the injection of labelled myoblasts into different muscle environments, such as developing, diseased, regenerating and/or undamaged muscle. Although these injection schemes have proved useful, there are several limitations to the experiments. First, the efficient transfer of foreign myoblasts into a host animal poses a problem with immune rejection (Labrecque *et al.* 1992; Huard *et al.* 1992). Although there is evidence that immune suppression is not necessary for the survival of the implanted myoblasts in syngeneic hosts (Rando and Blau, 1994), there is mounting evidence that the use of immunosuppressive drugs such as FK506 (Kinoshita *et al.* 1994) and cyclosporin (Labrecque *et al.* 1992) may be necessary for the survival of injected myoblasts. Recent evidence suggests that only transient suppression of the immune system is necessary for the long term survival of the implanted cells (Watt *et al.* 1984; Pavlath *et al.* 1994). While some immunosuppression may be essential for maintaining muscle graft, such immune suppression may lead to the production of tumours (Wernig *et al.* 1991).

Another obstacle to successful myoblast transplantations is the problem of identifying donor myoblasts in the injected muscle. While the use of retroviral vectors and fluorescent dyes has been useful in determining fibres that contain donor cells, the spread of these dyes often prevents the specific identification of donor nuclei within a fibres (Ralston and Hall, 1989; Ralston and Hall, 1989). Specific labelling of nuclei through radioactively - labelled molecules (Karpati *et al.* 1989) and thymidine analogues such as BRDU (Labrecque *et al.* 1991), allow for donor nuclei identification, but these labels are diluted out by repeated cell division. Finally, the injection of cells usually is preceded by extensive culturing of the myoblasts to both expand and label the cells. This extensive culturing may adversely effect the cells and therefore, eliminate any possible intrinsic programming of the

cells.

Recently, a group of investigators have started injecting modified myoblasts and myotubes into brain of rat (Jiao and Wolff, 1992). The myoblasts were genetically altered to produce and secrete growth factors that would assist in the repair of brain disorders found in rat models of Parkinson's disease (Jiao *et al.* 1992). The myoblasts that were injected fused to form large myotubes that could still be observed up to 56 days after injection in both the cerbral cortex and caudate-putamen. An important point to note is that the animals did not require immunosuppressive drugs to maintain the grafts, suggesting that the blood-brain barrier becomes re-established after injection and prevents the donor cells from being recognized as foreign. Such a transplantation scheme has several advantages. As already mentioned, no immunosuppressant drugs need to be used which would make the rats susceptible to infection. Secondly, myoblasts need not be labelled since there is no endogenous muscle in the brain. Finally, extensive culturing is not necessary prior to injection. Therefore, any program that has been established during development is more likely to be expressed upon injection.

By using the brain as an environment for myoblast proliferation, fusion and maturation, one can address the role of both growth factors and neurotrophic factors on muscle development. Following brain injury specific glial cells, known as astrocytes, become activated and are known to secrete a plethora of ubiquitous growth factors into the surrounding tissue. Several of these growth factors, including fibroblast growth factor (FGF) (Garcia-Estrada *et al.* 1992), transforming growth factor β (TGFβ) (Lindholm *et al.* 1992) and insulin growth factor (IGF) (Logan *et al.* 1992), have been shown to have a profound effect on the proliferative ability of myoblasts *in vitro* (Gospodarowicz and Mescher, 1977; Heino and Massagué, 1990; Zentella and Massagué, 1992; Florini *et al.* 1991; Ewton and Florini, 1990), and are present during morphogenesis of the hindlimb during development (Jones, 1980; McLennan, 1993). Since the cells are located in the middle of the central nervous system, the effects of putative neurotophic agents and possible innervation may also

be examined. The state of muscle innervation can be examined through the distribution of several different surface receptors, including the acetylcholine receptor (AchR) (reviewed by Fambrough, 1979)) and neural cell adhesion molecules (NCAM) (Fredette *et al.* 1993).

Initially NCAM first appears on the surface of myoblasts prior to fusion (Moore *et al.* 1987; Covault and Sanes, 1986; Lyons *et al.* 1992). It has been suggested that these surface receptors help in the fusion process through homophilic binding between adjacent cells (Salmons and Jarvis, 1992; Rutishauser *et al.* 1995). The levels of NCAM then decrease slightly, then increase to be localized along the entire surface of the myotube membrane (Covault and Sanes, 1986). The NCAM receptors at this point are believed to assist in the formation of neuromuscular junctions through similar homophilic interactions (Rutishauser *et al.* 1995; Grumet *et al.* 1982). The addition of NCAM-specific antibodies into muscle/nerve cultures blocks the formation of synapses between the two populations (Remsen *et al.* 1990; Bixby *et al.* 1987). Once a synapse has been formed and becomes functional, the NCAM receptors disappear from the majority of the fibre's membrane, and become specifically localize to the sub- and perisynaptic membrane of the myotube (Covault and Sanes, 1986). Following denervation, there is a distinct increase in the levels of NCAM along the entire surface of the muscle (Covault and Sanes, 1985; Covault *et al.* 1986). Similar increases in NCAM are found in regenerating (Irintchev *et al.* 1994) as well as in the muscle of older animals (Andersson *et al.* 1993), all of which involve an increase in the number of denervated muscle fibres. Also, allowing reinnervation of the muscle to occur results in the rapid down-regulation of NCAM similar to that seen during development (Covault and Sanes, 1985). Therefore, by following the distribution of NCAM on the myotubes surface, it is possible to determine the state of innervation.

Since the introduction of a needle into the brain will initiate processes that in some ways mimic the environment found during myogenesis (ie. production of high amounts of growth factors and sprouting axons), this offers an ideal location to study

the differentiation of the embryonic and fetal cell populations over a long period of time. To determine if two myogenic lineages exist during myogenesis of the hindlimb, the individual populations were injected into the caudate putamen of adult rats and their fate was followed for four weeks after injection using the panel of Mabs specific for MyHC isoforms. To determine the level of maturity that the resulting myotubes obtained, they were examined for the presence of peripherally-located nuclei and cross striations. The state of innervation was also analyzed using a polyclonal antibody specific for all NCAM isoforms (Rougon and Marshak, 1986). These experiments indicate that mature muscle can be formed upon injection of either population and that a distinct myogenic lineage of slow MyHC-expressing myoblasts may be present at only early time points in development.

4.2 Methods and Materials

4.2.1 Myoblast Injections into the Caudate-Putamen

Primary myoblasts were obtained from either ED 14 hindlimb buds and adjacent trunk (embryonic cell injections) or from ED 20 hindlimbs (fetal cell injections) as previously described in section 2.2.1. Twenty-four hrs after plating, the cells were trypsinized as described in section 2.2.3. Following trypsinization, harvested cells were washed once with cold complete media (defined in Section 2.2.3) and then twice with Ca^{++} Mg^{++} Free Hank's Buffered Salt Solution (CMF-HBSS; Gibco/BRL, Burlington, Ont). Prior to injection into the caudate-putamen, cells were resuspended at a concentration of 50,000 cells / µl of CMF-HBSS with 0.1% India ink (Pelikan, Hannover, Germany).

For each injection experiment, ten Sprague Dawley rats (Charles River, Montreal, Que), between the ages of two to three months, were anesthetized with intraperitoneal injections of sodium pentobarbital (60 mg/kg body weight; M.T.C. Pharmaceuticals, Cambridge, Ont), and then placed in a stereotaxic frame. The skin and underlying muscle were retracted to expose the cranium over the predetermined area of the injection site. Using a surgical drill, small burr holes were carefully made

on both sides just anterior to the bregma. The dura was then excised to allow free access to the brain. Cells were then delivered stereotaxically into the caudate-putamen, using either a 10 or 25 µl Hamilton syringe (Fisher Scientific, Unionville, Ont) mounted on a stereotaxic tower. Cells were injected at a rate of one µl/min. In all, 10 µl (containing 500,000 cells) of the cell suspension was delivered in ten min. Following delivery of the cell suspension, the needle remained in place for three min in order to allow the cells to be properly dispersed. Cell injections were repeated in an identical fashion on the contralateral side, such that each rat received two cell injections. Following removal of the needle, any bleeding was stopped and the skin was sutured back in place using surgical staples. Animals were then allowed to recover overnight under heat lamps, and returned to solitary, sanitary conditions after 24 hrs. All rats received two injections of the analgesic, buprenorphine (Temgesic) - one immediately after cell injection, and a second twenty-four hrs later

4.2.2 Identification of Cell Injection Sites

Two rats which received embryonic cell injections, were sacrificed at 7 days after injection, while four rats were sacrificed at 14 and 28 days after injection. Rats receiving fetal cells were sacrificed at 7 and 14 days (two rats each), 28 days (four rats), or 56 days (two rats) post-injection. At these time points, the rats were temporarily anesthetized by inhalation of halothane (Halocarbon Labs, North Augusta, SC) and then guillotined. Fresh brains were dissected out of the skull after the surrounding bone was removed, and then embedded in Tissue Tek OCT (Miles Inc., Elkhart, In.) freezing compound upon immersion in melting isopentane (BDH Inc, Toronto, Ont). Each block was serial sectioned at 10 - 15 µm on a Leitz cryostat, and the resulting sections were placed onto gelatin coated slides (gelatin; Sigma Chemical Co, St Louis, MO). These sections were stored at -20°C until their MyHC expressional profiles could be examined. Sections were analyzed for MyHC expression if an injection site could be identified either by the presence of India ink, or by the obvious appearance of aberrant tissue in the brain.

4.2.3 Characterization of Embryonic and Fetal Muscle Tissue in the Caudate-Putamen

To determine the distribution of muscle within the brain, every tenth section was tested for embryonic MyHC (47A) and neonatal/adult fast MyHC (MY-32), using indirect immunofluorescence co-localization, as previously described in section 2.2.7. Areas in which substantial amounts of muscle could be observed were further analyzed for MyHC expression. Initially, serial sections were examined with ABC-AP immunohistochemistry, as described in section 2.2.1, using antibodies specific for embryonic MyHC (47A), neonatal/adult fast MyHCs (MY-32), fast IIA MyHC (SC.71 and 4A.74), fast IIB MyHC (BF.F3), fast IIB/IIX MyHCs (212F), slow MyHC (8H8, 10D10, 4A9, and 4A.951), and all isoforms except embryonic and fast IIX MyHC (BF.35). From these localizations, a general MyHC expression profile was obtained for each population of cells.

4.2.4 Characterization of Individual Myotubes using Immunofluorescent Localization

To determine the phenotypic profile of individual myotubes in both the embryonic and fetal cell populations, fluorescent double localizations, essentially similar to those previously described in section 2.2.7, were used. In all cases, 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont) was used to block sections for 30 min at 37°C. The first primary incubation consisted of Mabs belonging to the either the IgG_{2A} class, which included 47A (diluted 1 in 10 in BSA-PBS), 10D10 (1:5), and 8H8 (1:25-1:50), or BF.F3 (undiluted), which belongs to the IgM class of antibodies. Following an incubation of 45 min and several washes in phosphate buffered saline (PBS), sections were incubated in a fluorescein-conjugated rabbit anti-mouse (FITC-RAM) IgG_{2A} secondary antibody (diluted to 1 in 50; ICN Biomedicals Canada Ltd, Montral, Que) for 45 min and then washed again. Mabs belonging to the IgG_1 class, including MY-32 (1:100), 212F (1:3), 4A.74 (undiluted), SC.71 (undiluted), and 4A.951 (undiluted) were reacted in a similar way and detected with a RITC-SAM

IgG_1 antibody at 1:50. Following incubation of the second fluorescent antibody, slides were washed with PBS and coverslipped with a glycerol - based mountant (containing 5% paraphenyldiamine: BDH Inc, Toronto, Ont).

4.2.5 Analysis of NCAM Expression in Embryonic and Fetal Cell Injections

To determine if innervation could be correlated with MyHC expression, embryonic and fetal cell injection sites were analyzed using triple localizations of a polyclonal antibody specific for all NCAM isoforms (Rougon and Marshak, 1986), with various combinations of MyHC specific Mabs. The procedure was essentially the same as that described for immunofluorescent double localizations, except that the first set of incubations with MyHC Mabs was preceded by an incubation of the NCAM polyclonal antibody (diluted 1 in 500) followed by a goat-anti-rabbit secondary antibody conjugated to Cy5 (GAR-Cy5 at a dilution of 1 in 75; Jackson ImmunoResearch Labs Inc, West Grove, PA). In all cases, fluorescent localizations were analyzed and images obtained using a Zeiss LSM 410 confocal microscope. 10 to 15 µm sections were optically sectioned into 0.7 µm sections and then projected into one plane to preserve the focus of the images. Prints were produced using a Phaser 440 Tektronic dye sublimation printer.

4.3 Results

4.3.1 Characterization of Embryonic and Fetal Cell Injection Sites in the Caudate-Putamen

The injection of fetal or embryonic cells into the caudate-putamen of adult rats resulted in injection sites that were vastly different with respect to their size, muscle organization, and overall developmental potential (Figure 4.1). Injection of fetal cells resulted in small injection sites that at seven days were confined to the periphery of the brain (ie. in the cerebral cortex) or to the needle tract. The limits of injection sites appeared to reduce over time, and by 28 days, injection sites resembled only thin tracts. The muscle that was present showed very little organi-

Figure 4.1 Morphological comparison of embryonic and fetal cell injection sites in the caudate-putamen to fetal hindlimb. Grafts were examined for their general level of organization and differentiation after staining with methylene blue. Individual muscle masses can be distinguished in fetal hindlimbs from ED 20 (A). These muscles surround two osteogenic cores which represent the tibia and fibula. In embryonic injection sites (B), a similar pattern of organization can be observed with several muscle fascicles (seen in the enlarged area) surrounding a osteogenic core (⇦). In contrast fetal injection sites (C) show relatively small accumulations of muscle cells and no higher levels of organization. Bar = 465 μm; for insert = 115 μm.


A
B
C

zation, running longitudinally inside the injection tract. These myotubes were surrounded by very little connective tissue, and necrosis was apparent. By 56 days, no cells were observed, presumably due to either a lack of nutrients reaching the myotubes or as a result of rejection by the host. In contrast, embryonic cell injections produce large injection sites that showed extensive proliferation, which ended up occupying the majority of the caudate-putamen as well as some adjacent areas. Large fascicles of muscle could be observed in which the myotubes exhibited peripherally-located nuclei suggesting that they had become mature muscle fibres. Surprisingly, these fascicles surrounded central cores containing calcifying cartilage. Closer examination of the cartilage revealed the presence of bony trabeculae, indicating a possible epiphyseal growth plate (data not shown). Although there was a large amount of connective tissue and collagen interspersed between the fascicles, the overall appearance of the injection sites was very similar to developing fetal hindlimbs.

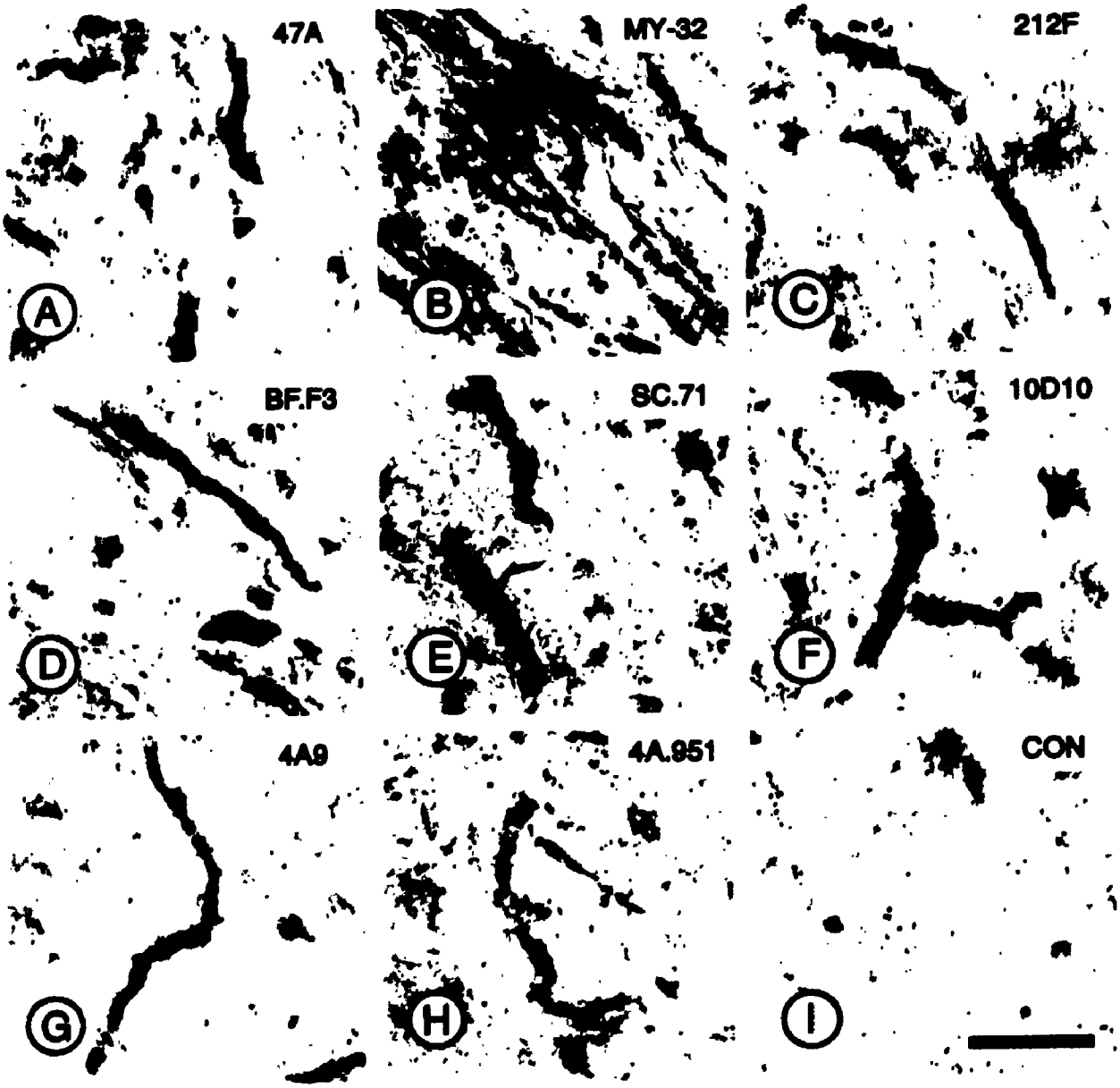
To determine the phenotypic profile of the myotubes present in fetal injection sites, ABC-AP localizations using Mabs specific for the various MyHCs was performed at one, two and four weeks post-injection (Table 4.1). At one week post-injection, myotubes were recognized by both 47A and MY-32 but not by any of the antibodies specific for the adult fast MyHCs. This indicates that both embryonic and neonatal fast MyHCs were expressed early after cell injection. Interestingly, Mab 8H8 (specific for slow MyHC) reacted with this population of myotubes as well. However, all of the other slow MyHC - specific Mabs showed little reaction, suggesting a possible difference in their specificity, similar to the expression seen in fetal cell cultures (Chapter 2). ABC-AP localizations at 14 days after injection revealed the same phenotypic profile of MyHCs, with 47A (embryonic MyHC), MY-32 (neonatal/adult fast MyHC) and 8H8 (slow MyHC) the only Mabs that reacted positively. Twenty-eight days after injection (Figure 4.2), ABC-AP immunolocalization with Mab MY-32 still showed a strong reaction indicating the presence of neonatal/adult fast MyHC isoforms. In addition to this isoform, positive

Table 4.1 Myosin heavy chain expression in fetal myoblasts after injection into the caudate-putamen of adult rats

MyHC Isoform	Weeks Post - Injection		
	One Week	Two Weeks	Four Weeks
Embryonic (47A)	+++	+++	++
Neonatal (MY-32, NN6)	++	+++	+++
Adult Fast IIA (SC.71, 4A.74)	-	-	+
Adult Fast IIB (BF.F3)	-	-	+
Adult Fast IIX (212F)	-	-	++
Embryonic Slow (8H8)	+	+	+
Neonatal Slow (4A9, 10D10)	-	+	+
Adult Slow (4A.951)	-	-	+

+ - less than 30% of the observed myotubes
++ - less than 90% of the observed myotubes
+++ - greater than 90% of the observed myotubes

Figure 4.2 Immunolocalization of MyHC isoforms in fetal myoblasts after injection into the caudate-putamen of adult Sprague Dawely rats. Fetal myoblasts were examined for MyHC expression at 28 days post - injection using ABC - AP immunohistochemistry using Mabs 47A (A), MY-32 (B), 212F (C), BF.F3 (D), SC.71 (E), 10D10 (F), 4A9 (G), and 4A.951 (H). These myotubes are positive for embryonic (A; 47A) and neonatal/adult fast (B; MY-32) MyHCs. Myotubes expressing IIB/IIX (C;212F), IIB MyHC (D;BF.F3), and IIA (E;SC.71) were also detected. Smaller subsets of myotubes also reacted positively to slow - specific Mabs including 10D10 (F), 4A9 (G) and 4A.951 (H). The ABC-AP reaction carried out with no primary antibody (I) shows only a light background with no specific staining. Bar = 45 μm.


47A
MY-32
212F
A
B
C
BF.F3
SC.71
10D10
D
E
F
4A9
4A.951
CON
G
H
I

reactions were also observed for Mabs 212F (IIB/IIX), 4A.74 (IIA), SC.71 (IIA), and BF.F3 (IIB), indicating that all of the adult fast MyHCs are expressed. Further examination of these injection sites also revealed positive reactions for all of the slow Mabs (8H8, 10D10, 4A9 and 4A.951). These unexpected results indicate that the ED 20 population of cells produced myotubes which expressed all MyHC isoforms, including adult slow isoforms. Interestingly, while Mab 47A still reacted positively to the fetal myotubes, the level of reactivity was low indicating that the embryonic MyHC may not be expressed at its initially high levels.

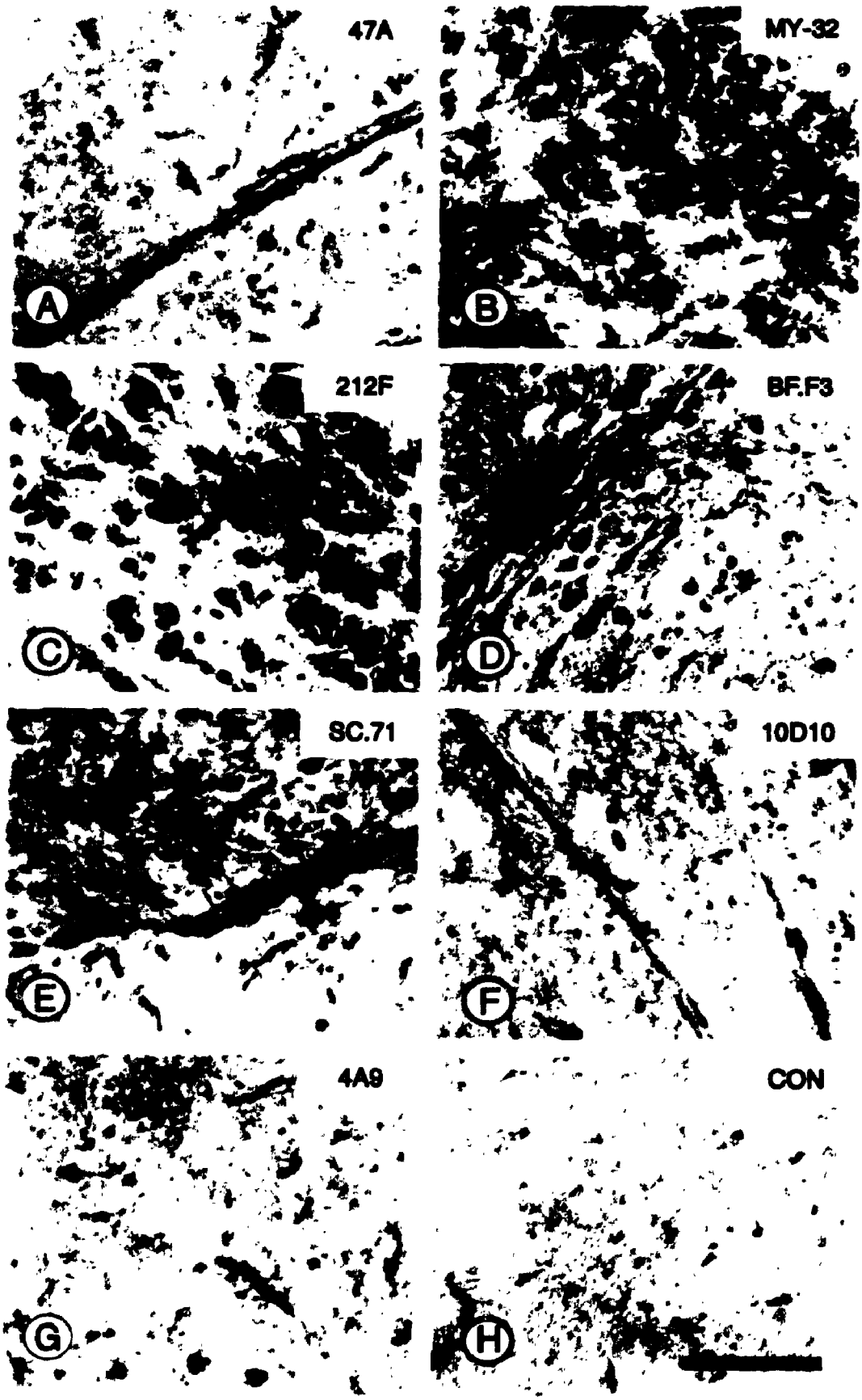
To determine if embryonic cells exhibited a similar MyHC profile, similar ABC-AP immunolocalizations were performed on embryonic myoblast injection sites at seven, 14, and 28 days after injection. These myotubes exhibited a similar pattern of expression to the fetal myotubes at seven days post-injection (Table 4.2), reacting positively for 47A and 8H8 (both of which recognized these cells in culture) as well as MY-32 (which did not). By 14 days after injection, 10D10 and 4A9, as well as 4A.951 to a lesser extent, recognized some of the embryonic myotubes. In contrast, none of the fast MyHC specific Mabs showed a positive reaction to these myotubes. At 28 days post-injection ABC-AP immunolocalization (Figure 4.3) revealed strong staining for all slow antibodies, including 8H8, 4A9, 10D10 and 4A.951. Surprisingly, Mabs BF.F3, 212F, 4A.74 and SC.71 all reacted to a subset of the myotubes, indicating the presence of IIB, IIA, and possibly IIX MyHC. Once again, 47A showed a much weaker reaction when compared to earlier time points, and some myotubes observed did not stain for 47A at all, indicating that this isoform may be down-regulated. The fact that both populations of cells exhibited staining for all the developmental and adult MyHC isoforms suggests that, in general, they have similar developmental potentials, and that these potentials are not fully realized in culture conditions.

Table 4.2 Myosin heavy chain expression in embryonic myoblasts after injection into the caudate-putamen of adult rats

MyHC Isoform	Weeks Post - Injection		
	One Week	Two Weeks	Four Weeks
Embryonic (47A)	+++	+++	++
Neonatal (MY-32, NN6)	++	+++	+++
Adult Fast IIA (SC.71, 4A.74)	-	-	+
Adult Fast IIB (BF.F3)	-	-	+
Adult Fast IIX (212F)	-	-	++
Embryonic Slow (8H8)	+	+	+
Neonatal Slow (4A9, 10D10)	-	+	+
Adult Slow (4A.951)	-	+	+

+ - less than 30% of the observed myotubes
++ - less than 90% of the observed myotubes
+++ - greater than 90% of the observed myotubes

Figure 4.3 Immunolocalization of MyHC isoforms in embryonic myoblasts after injection into the caudate-putamen of adult Sprague Dawely rats. Embryonic myoblasts were examined for MyHC expression at 28 days post - injection using ABC - AP immunohistochemistry. Many of these myotubes are positive for embryonic (A; 47A) and neonatal/adult fast (B; MY-32) MyHCs. Myotubes expressing IIB/IIX MyHC (D;212F), IIB MyHC (C;BF.F3), and IIA MyHC (E;SC.71) were also detected. Smaller subsets of myotubes also reacted positively to slow - specific Mabs including 10D10 (F) and 4A9 (G). The ABC-AP reaction carried out with no primary antibody (H) shows only a light background with no specific staining. Bar = 70 μm.


47A
A
MY-32
B
212F
C
BF.F3
D
SC.71
E
10D10
F
4A9
G
CON
H

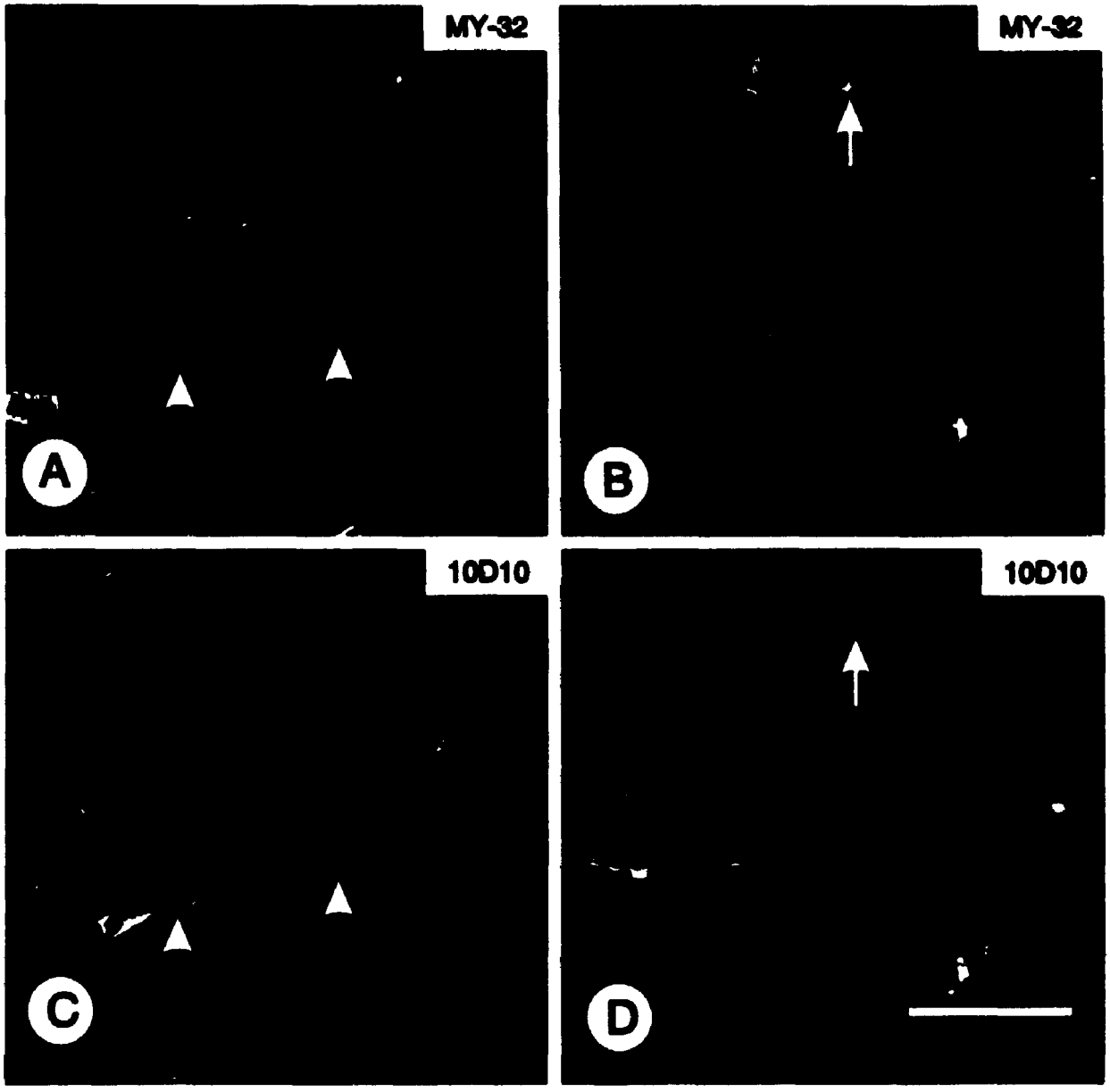
4.3.2 Characterization of Fast and Slow MyHC Isoforms in Fetal and Embryonic Cell Injections

Since myotubes expressing all of the MyHC isoforms were observed in both fetal and embryonic cell injection sites after four weeks, initial results indicated that both myoblast populations had the same developmental phenotype. However, upon closer examination, fluorescent localizations revealed a population of embryonic myotubes that expressed only slow MyHC. Myotubes expressing exclusively slow MyHC were not observed in fetal cell injections. Similar studies co-labelling Mabs specific for individual adult fast MyHCs with slow MyHC Mabs indicated that slow MyHCs can be co-expressed in myotubes that express adult fast MyHCs. However, this seemed to be restricted to the co-expression with fast IIA MyHC, indicating that an internal program may have been established prior to full maturation of the myoblasts, limiting their MyHC expression.

To determine the MyHC expression of individual myotubes, Mabs specific for neonatal/adult fast MyHC (MY-32) and slow MyHC (10D10) were colocalized and sequentially recognized by different fluorescent-conjugated isotype-specific secondary antibodies. The characterization of fetal and embryonic injection sites 14 days after injection using this method revealed differences between the two cell populations (Figure 4.4). Uponco-labelling of 10D10 and MY-32 in fetal cell injections, all identified myotubes either reacted to MY-32 only or both MY-32 and 10D10. This suggests that there are two general populations of fibres present in the fetal cell population; a large population of fast only myotubes, and a second smaller population of fast/slow myotubes. Similar labelling techniques done in the embryonic injection sites produced a different picture, with myotubes recognized by 10D10 only, MY-32 only, or both Mabs. The presence of myotubes labelled only for 10D10 suggests that a third population, expressing only slow MyHC and restricted to early time points in development, may formed by a myoblast lineage present only in the embryonic cell population.

To ascertain if the difference in fibre types between these populations is tran-

Figure 4.4 Confocal microscopy showing the co-localization of neonatal/adult fast and slow MyHCs in embryonic and fetal cells at 14 days after injection into the caudate-putamen. Myotubes formed by embryonic cells (A,C) and fetal cells (B,D) were analyzed with antibodies which recognize neonatal/fast MyHCs (MY-32;A,B) or slow MyHC (10D10;C,D). Fast and slow Mabs were recognized by isotype specific secondary antibodies conjugated to rhodamine and fluorescein, respectively. Analysis of embryonic myoblast injection sites reveals myotubes that express only slow MyHC (▲), only fast MyHC (→) or both slow and fast MyHCs (seen in both panels). Fetal injection sites characterized in a similar manner (B,D) show the presence of myotubes expressing only fast or both fast and slow MyHCs. No fetal myotubes were observed to express only slow MyHC. Bar = 45 μm for A and C, and 40 μm for B and D.


MY-32
A
MY-32
B
10D10
C
10D10
D

sient, identical fluorescent localizations were performed on injection sites 28 days post-injection (Figure 4.5). Once again characterization of fetal myotubes revealed the presence of only two populations of myotubes - fast only (recognized by MY-32 only), and fast/slow (recognized by both MY-32 and 8H8). Myotubes present in embryonic injection sites could still be categorized into fast (MY-32 positive), slow (8H8 positive) and fast/slow (MY-32/8H8 positive) populations. However, the actual percentage of slow only fibres dropped from 17.8% at two weeks post-injection to 3.5% at four weeks post-imjection (Table 4.3), indicating that either the slow fibres were gradually changing into fast/slow myotubes, or that the other populations have increased due to preferential addition of new fast and fast/slow myotubes between 14 and 28 days post-injection. Alternatively, the large increase in the fast/slow population may indicate that fusion between the fast only and slow only populations may be occurring, especially since the percentage of fast only myotubes is also decreasing.

Although myotubes can be shown to react to both MY-32 and 10D10 at 28 days after injection, this does not shed light on the possible co-expression of adult slow and adult fast MyHCs, an occurrence which is rare *in vivo*. Since the determination of fast and slow MyHC double localization depends on the availability of Mabs with different isotype specificities, it was important to determine if 10D10 and 8H8, which are IgG_{2A} Mabs, are coextensive with 4A.951, which is an IgG_1 Mab and known to be specific for the adult slow isoform (Hughes *et al.* 1993). It is important to prove such a correlation since all of the available antibodies specific for adult fast MyHC are IgG_1 Mabs, with the exception of BF.F3, which is an IgM Mab. Direct labelling of primary antibodies would not provide sufficient sensitivity for the detection of the various MyHCs. Since previous studies suggested that the slow antibodies had different specificities, these antibodies were colocalized on both fetal and embryonic injection sites at various time points after injection. Although there were differences in reactivity early after injection (such as 8H8 positive myotubes not being recognized by Mabs 10D10, 4A9, and 4A.951; data not shown) these Mabs

Figure 4.5 Confocal microscopy showing the co-localization of neonatal/adult fast and slow MyHCs in embryonic and fetal cells at 28 days after injection into the caudate-putamen. Myotubes formed by embryonic cells (A,C) and fetal cells (B,D) were analyzed with antibodies which recognize neonatal/fast MyHCs (MY-32;A,B) or slow MyHC (10D10;C,D). Fast and slow Mabs were recognized by isotype specific secondary antibodies conjugated to rhodamine and fluorescein, respectively. Analysis of embryonic myoblast injection sites still shows the existence of a population of myotubes which express only slow MyHC (▼), as well as populations which express only fast or fast and slow MyHCs. Fetal injection sites characterized in a similar manner (B,D) show the presence of myotubes expressing only fast or both fast and slow MyHCs. No fetal myotubes were observed to express only slow MyHC. Bar = 70 μm and A and C, and 55 μm B and D.

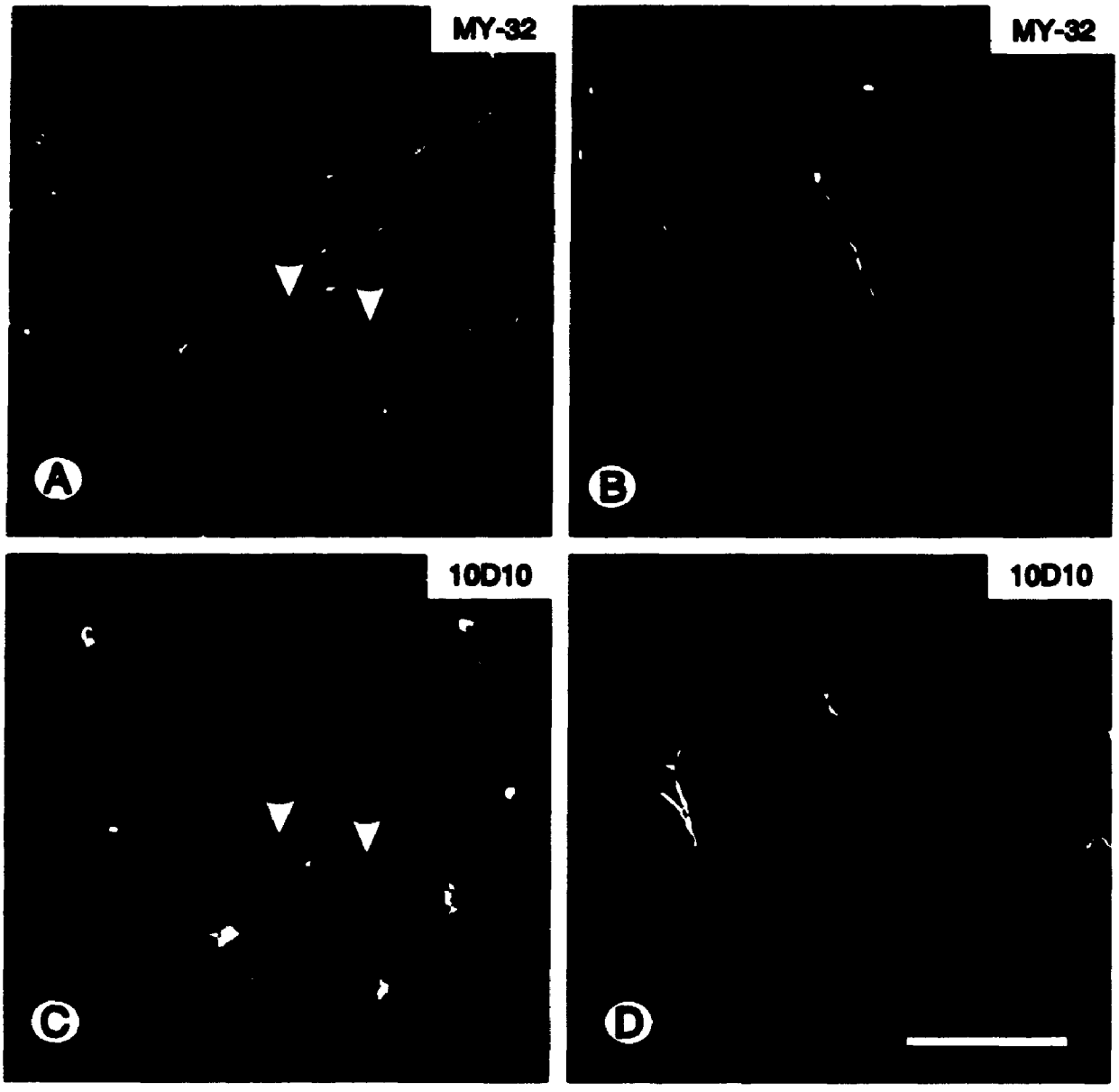

MY-32
A
MY-32
B
10D10
C
10D10
D

Table 4.3 Expression of fast and slow myosin heavy chain isoforms in embryonic cell injections into the caudate-putamen

Monoclonal Antibody	Two weeks post-injection		Four weeks post-injection	
	# of myotubes	% of myotubes	# of myotubes	% of myotubes
MY-32	257	76.3	337	66.1
10D10	60	17.8	18	3.5
MY-32 / 10D10	20	5.9	155	30.4
Total # of myotubes	337	-	510	-

were coextensive in both fetal and embryonic myotube populations by 28 days post-injection (Figure 4.6). This would suggest that at the time when the adult fast isoforms are being expressed, all of the Mabs specific for slow MyHC recognized myotubes expressing adult slow MyHC. Therefore it is possible to determine which, if any, of the fast MyHC isoforms can be colocalized with slow MyHC.

To examine the potential of slow MyHC expressing myotubes that also react to MY-32, Mabs 10D10 (specific for slow MyHC) and either 212F (IIB/IIX), 4A.74 (IIA), and SC.71 (IIA) were co-labelled and recognized with fluorescent-conjugated isotype-specific secondary antibodies. Due to the lack of reactivity of the fast MyHC Mabs at earlier time points, double localization were carried out for fetal and embryonic cell injections at 28 days post-injection only (Figure 4.7). Co-labelling of 4A.74 and 10D10 revealed myotubes recognized by both Mabs indicating the co-expression of fast IIA and slow MyHC. Closer examination of the fetal cell injections revealed that all of the slow MyHC - expressing myotubes were also recognized by 4A.74 and therefore co-express IIA MyHC. Although many myotubes express both IIA and slow MyHC in the embryonic cell injections, as indicated by MY-32 labelling, there was also a population of slow myotubes that did not recognize 4A.74, and, therefore did not express IIA MyHC. In sharp contrast to these results, double localization of 10D10 and 212F revealed that the subsets of myotubes recognized by these Mabs are mutually exclusive, indicating that the slow MyHC isoform does not colocalize with fast IIB/IIX MyHC. This absence of slow myotubes recognizing IIB or IIX MyHC was observed for both the fetal and embryonic cell populations.

The difference between the two cell populations, with slow MyHC only fibres observed in embryonic cell injection sites only, suggests that there may be intrinsic differences between the two populations of myoblasts and that they may represent different myogenic lineages. Furthermore, the observation that only the fast IIA MyHC is colocalized with slow MyHC, suggests the existence of several myogenic lineages within each population.

Figure 4.6 Confocal microscopy showing the co-localization of slow Mabs 28 days after injection of embryonic (A, B, C, D) or fetal (E, F,G, H) cells into the caudate-putamen. 10D10 and 8H8 were both identified by an FITC-RAM IgG_{2A} antibody, while 4A.951 was recognized by a RITC-SAM IgG_1 antibody. Cryostat sections were analyzed with Mabs 8H8 (A,E), 4A.951 (B,D,F,H), and 10D10 (C,G). Labelling with 8H8 (A,E), which recognizes all slow isoforms, and 10D10 (C,G), which recognizes neonatal and adult slow isoforms, is coextensive with labelling for 4A.951. This suggests that all slow myotubes are expressing at least adult slow MyHC. Bar = 70 μm for A, B, C and D, 35 μm for E and F, 30 μm for G and H.

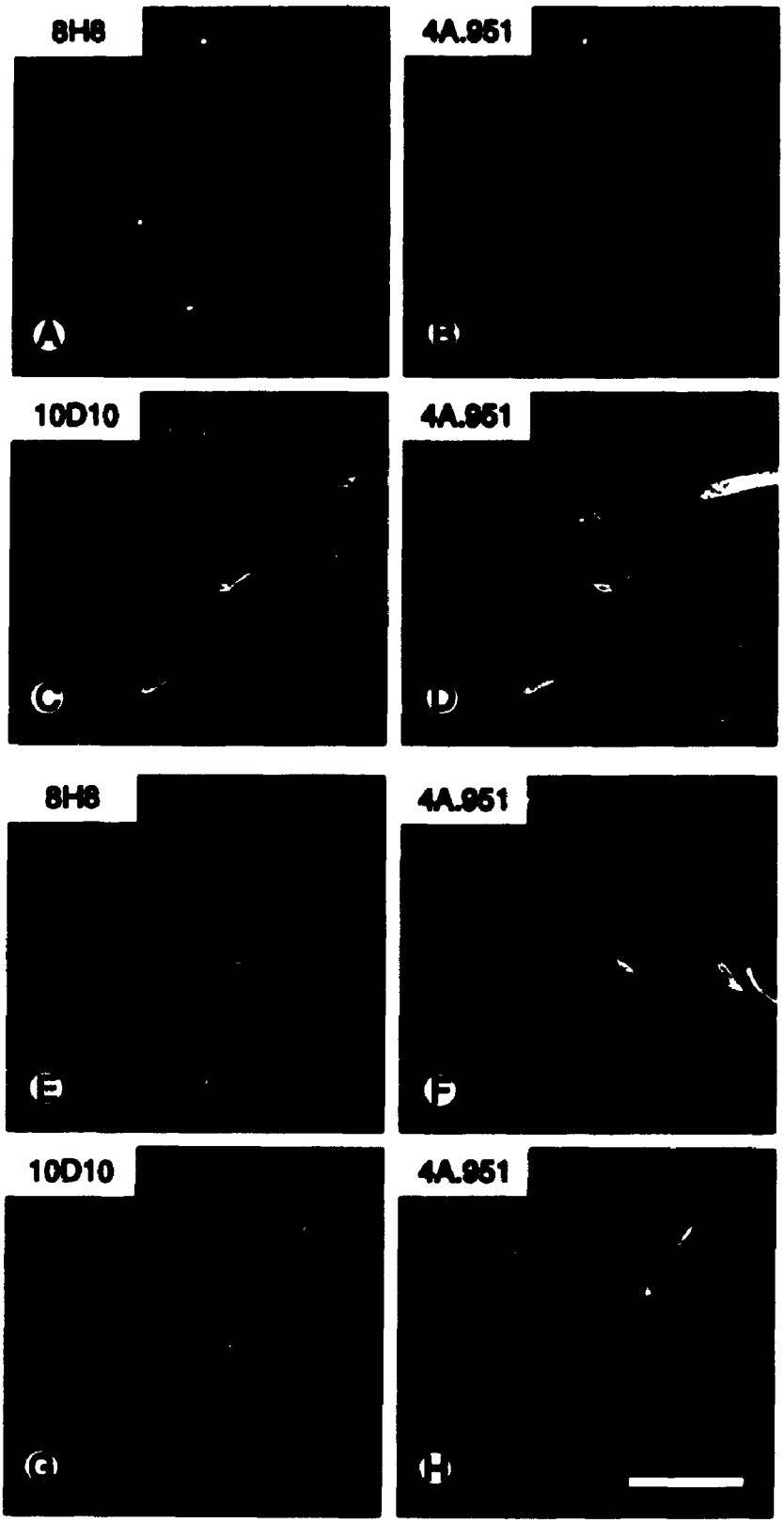
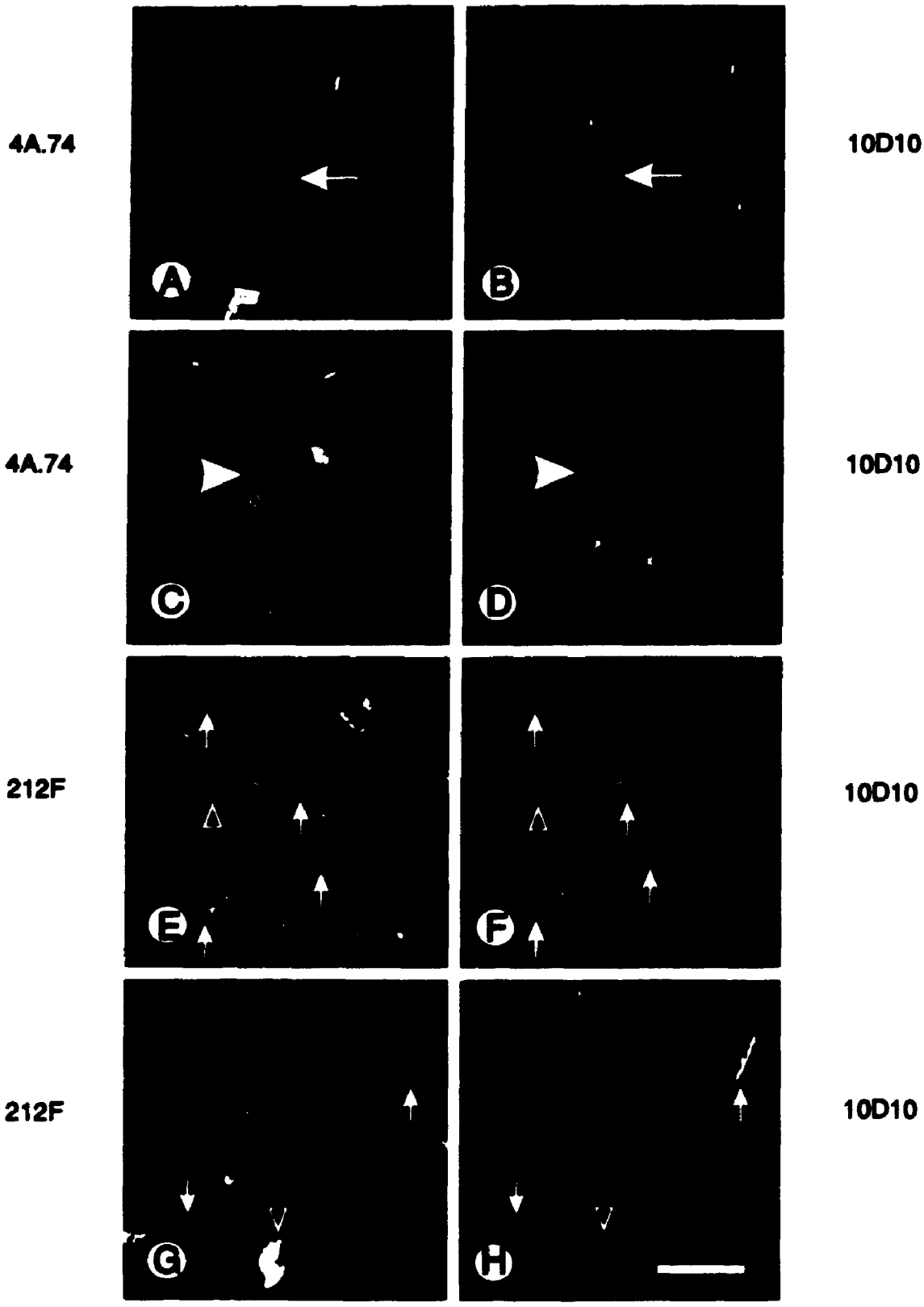

8H8
4A.951
(A)
(B)
10D10
4A.951
(C)
(D)
8H8
4A.951
(E)
(F)
10D10
4A.951
(G)
(H)

Figure 4.7 Confocal microscopy showing co-labelling of adult MyHC isoforms in embryonic (A, B, C, D) and fetal (E, F, G, H) myotubes 28 days after injection into the caudate-putamen. Sites injected with embryonic or fetal cells were analyzed for MyHC expression using indirect immunofluorescence with Mabs specific for IIA MyHC (4A.74; A,C), fast IIB/IIX MyHCs (212F; E,G), or slow MyHC (10D10;B,D,F,H). Mabs 4A.74 and 212F were detected using an RITC - SAM IgG_1, while 10D10 was colocalized with FITC - RAM IgG_{2A} secondary antibody. Myotubes which coexpress both slow and fast IIA MyHC (▲) are present in both embryonic (A,B) and fetal (C,D) injection sites. In contrast, slow and fast IIB/IIX MyHCs are not co-expressed, with embryonic (E,F) and fetal (G,H) myotubes expressing only slow (→) or only fast IIB/IIX (open arrow head) MyHCs. Bar = 35 μm for A and B, 50 μm for C and D, 30 μm for E and F, and 55 μm for G and H.


4A.74
10D10
A
B
4A.74
10D10
C
D
212F
10D10
E
F
212F
10D10
G
H

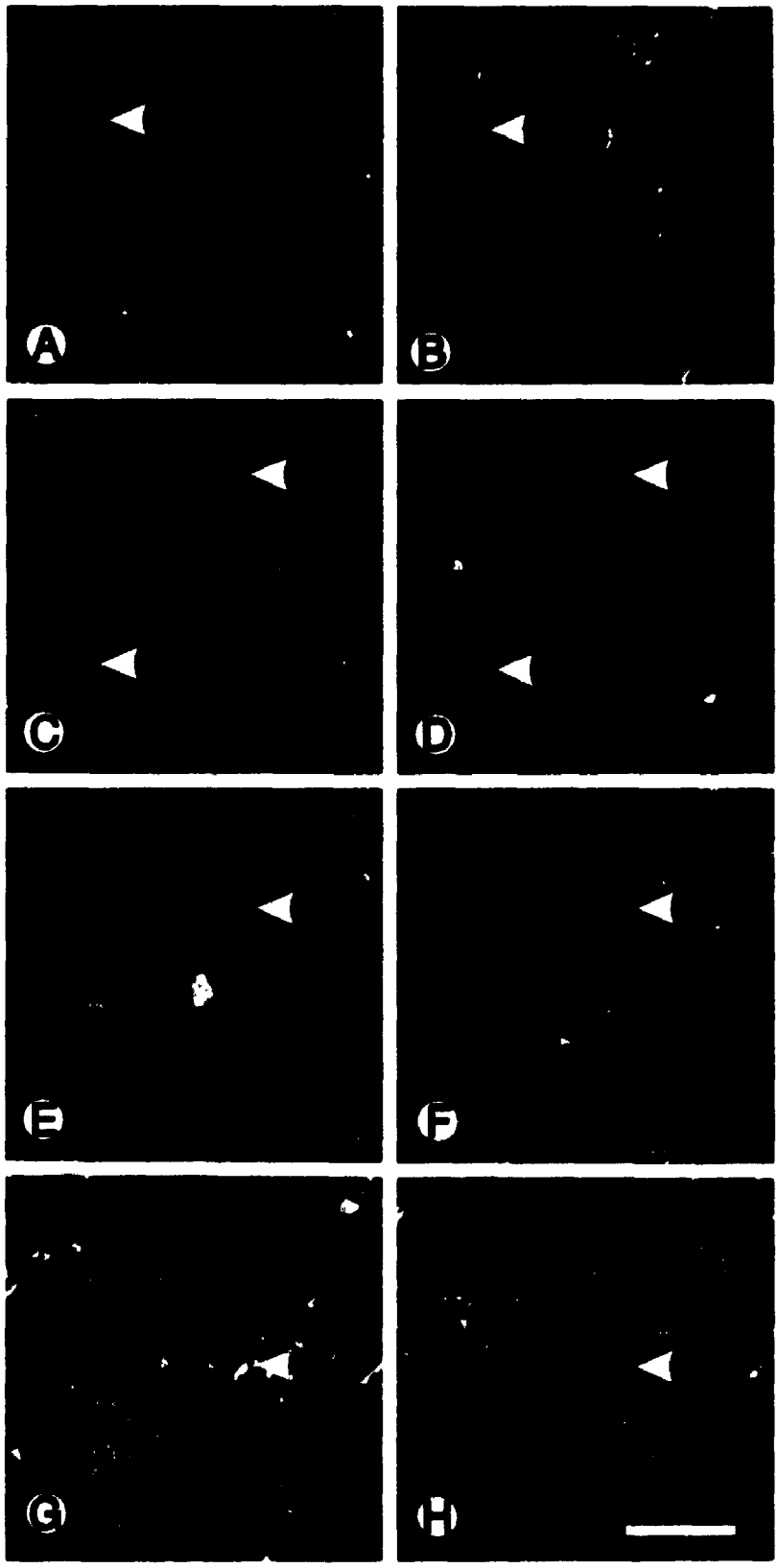
4.3.3 Co-expression of NCAM with MyHC Isoforms in Embryonic and Fetal Cell Populations

Without examining any possible environmental factors, it is impossible to conclude that the presence of different myotube populations (based on differential adult MyHC expression) is due to the presence of different myogenic lineages. To access the role that innervation may play in fibre typing, NCAM expression was analyzed within each myotube population. NCAM is only expressed prior to innervation and the lack of NCAM expression is indicative of innervated muscle cells.

To examine the possibility that myotubes within the injection site may be innervated, a polyclonal antibody specific for all NCAM isoforms was colocalized with Mabs specific for slow (10D10) and neonatal / adult fast (MY-32) MyHCs (Figure 4.8). Following the triple localization of antibodies on embryonic injection sites at 14 days post-injection, confocal microscopy revealed the presence of myotubes not labelled for NCAM indicating that these myotubes were innervated. Analysis of these myotubes for MyHC expression showed that both MY-32 and 10D10 recognized myotubes that had become innervated. However, the presence of myotubes that were both NCAM and 10D10 positive indicated that the expression of slow MyHC preceded innervation. These triple localizations were then carried out on 28 day injection sites. Once again, myotubes staining for 10D10 or MY-32, but not NCAM, were observed, indicating that innervated muscle cells express both slow and neonatal/fast MyHCs. In addition there are MY-32 and 10D10 positive myotubes which were also NCAM positive. The presence of 10D10 and NCAM positive myotubes also supports the hypothesis that slow MyHC expression precedes innervation. Since MY-32 and NCAM positive myotubes can be observed, this suggests that the expression of neonatal MyHC precedes innervation. This suggests that the differential expression of neonatal MyHC in injected cells compared to cultured cells is not due to innervation.

To examine the possibility that the adult fast MyHC isoforms might be upreg-

Figure 4.9 Confocal microscopy showing triple co-localizati f fast and slow MyHCs with NCAM in embryonic myotubes at 14 and 28 days after injection into the caudate-putamen. Myotubes were analyzed using triple co-localization with Mabs specific for slow (A,D;10D10) and fast (B,E; MY-32) MyHCs, with a NCAM polyclonal antibody (C,F). MyHC antibodies were recognized by either fluorescein (10D10) or rhodamine (MY-32) - conjugated isotype specific antibodies, while the NCAM polyclonal was recognized by a Cy5 - conjugated goat anti-rabbit secondary antibody. Confocal images of this localization at 14 days after injection (A,B,C) revealed the presence of both fast (▼) and slow (→) myotubes that are NCAM negative suggesting that they have become innervated. Similar co-labelling at 28 days (D,E,F) also revealed fast and slow myotubes that are NCAM negative, but the majority of myotubes still express NCAM. Bar = 30 μm for A, B, E and F, 25 μm for C and D, and 50 μm for G and H.


10D10
NCAM
212F
NCAM
4A.74
NCAM
BF.F3
NCAM
A
B
C
D
E
F
G
H

ulated by innervation, the NCAM polyclonal antibody was then localized with Mabs specific for the various adult MyHC isoforms. Double localization of NCAM and the adult MyHC specific Mabs in fetal cells 28 days after injection (Figure 4.9) revealed NCAM positive myotubes which also reacted with 10D10, 212F, 4A.74 or BF.F3, indicating the presence of the slow, IIA, IIB and possibly IIX MyHCs in fetal myotubes prior to innervation. Embryonic cell injection sites were also examined using triple localization with the NCAM specific antibody and Mabs specific for the various MyHCs at 28 days post-injection (Figure 4.10). Localizations of NCAM with 10D10 and either 212F, 4A.74 and SC.71 were analyzed with confocal microscopy, and confirmed observations made for the fetal myotubes. As already observed, 10D10 colocalized with 4A.74 and SC.71, but not 212F, again indicating the mutual exclusion of the fast IIB and IIX isoforms from the slow isoform. Myotubes that exhibit either 212F or 10D10 staining are both recognized by the NCAM antibody. The co-expression of the fast IIA MyHC with slow MyHC is also observed in NCAM positive myotubes.

To determine if other adult fast MyHC isoforms showed a similar restriction in their co-expression, a Mab specific for fast IIB MyHC (BF.F3) was localized with the NCAM specific polyclonal antibody and Mabs against either fast IIB/IIX (212F) or fast IIA (SC.71) MyHCs (Figure 4.11). Triple labellings were used to characterize embryonic cells 28 days after injection and examined by confocal microscopy. Upon analysis, all MyHC Mabs recognized NCAM positive myotubes. Triple labelling experiments revealed NCAM positive myotubes that reacted with BF.F3 and SC.71, indicating the co-expression of the IIA and IIB MyHCs. NCAM positive myotubes that were only recognized by one of the two Mabs were also detected suggesting that IIA, IIA/IIB and IIB myotubes developed prior to innervation. Labelling with BF.F3 and 212F revealed the presence of myotubes that were recognized by both Mabs or by 212F only. In both cases, these myotubes were NCAM positive, confirming the presence of IIX MyHC expression in the absence of innervation. Therefore, labelling of these myotubes with NCAM showed myotube maturation is not an effect of inner-

Figure 4.8 Confocal microscopy showing co-labelling of adult MyHCs with NCAM in fetal myotubes 28 days after injection into the caudate-putamen. Injection sites were analyzed using indirect immunofluorescence with Mabs specific for slow MyHC (A;10D10), IIB/IIX MyHCs (C;212F), IIA MyHC (E;SC.71) or IIB MyHC (G;BF.F3) being colocalized with an NCAM polyclonal antibody (B,D,F,H). The NCAM polyclonal antibody was recognized by a Cy5 - conjugated rabbit secondary antibody while the various MyHC Mabs were recognized by either fluorescein (10D10 and BF.F3) or rhodamine (212F and SC.71) - conjugated antibodies. Myotubes which express NCAM (and are therefore not innervated (▲)) express slow (A), IIB/IIX (C), IIA (E), and IIB (G) MyHCs. Bar = 17 μm A and B, and 50 μm for C and D.

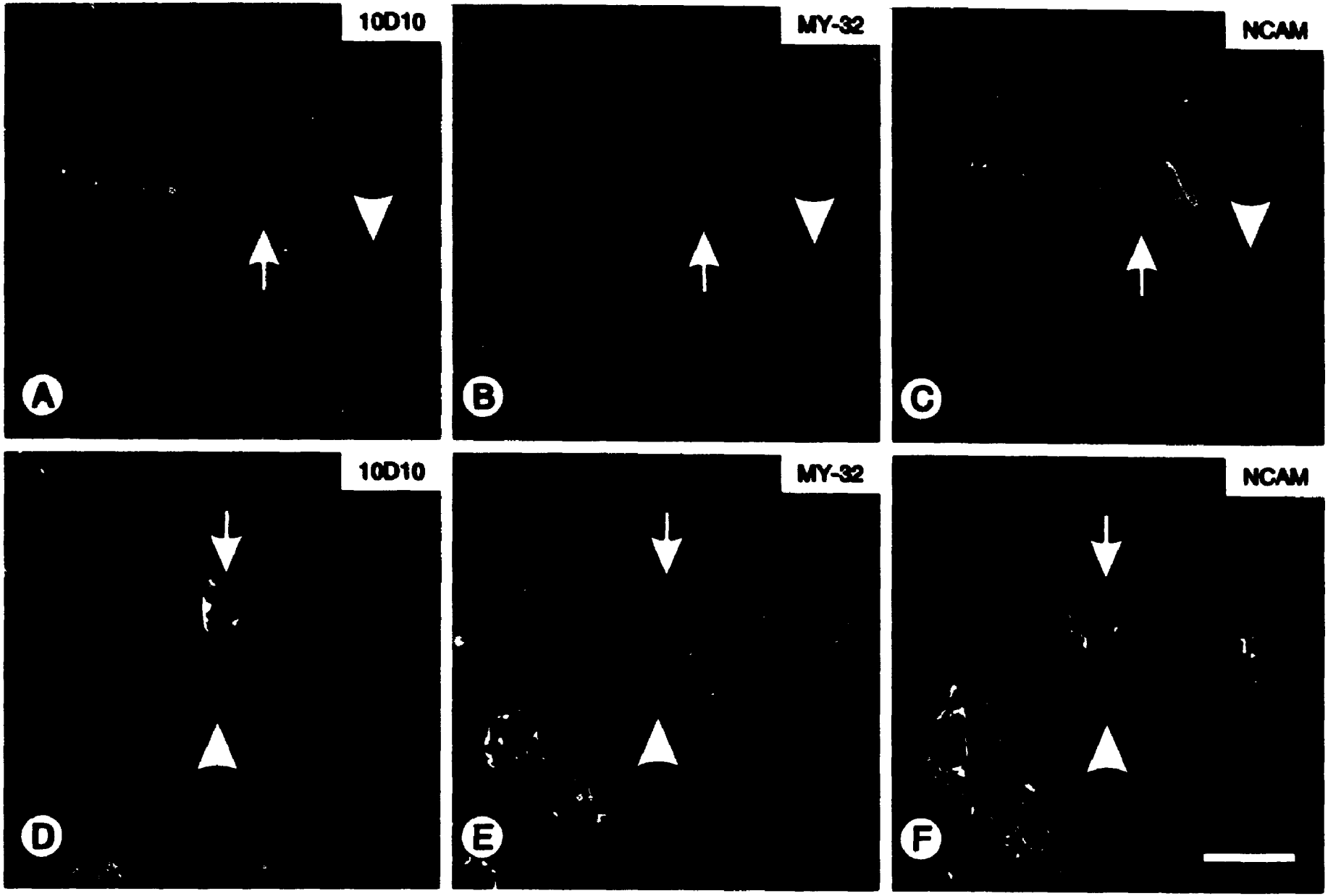

10D10
MY-32
NCAM
A
B
C
10D10
MY-32
NCAM
D
E
F

Figure 4.10 Confocal microscopy showing the co-expression of adult fast and slow MyHCs with NCAM in embryonic myotubes at 28 days after injection into the caudate-putamen. Sections were analyzed with antibodies specific for adult fast IIB/IIX (B;212F) and fast IIA (E;4A.74 and H;SC.71), colocalized with both a slow MyHC - specific Mab (A,D,G; 10D10) and an NCAM - specific antibody (C,F,I). Mouse Mabs against MyHCs were localized with isotype - specific secondary antibodies conjugated to either fluorescein (10D10) or rhodamine (212F, 4A.74, and SC.71). The NCAM - specific antibody was detected by a GAR-Cy5 conjugated secondary antibody. Triple labelling reveals NCAM positive myotubes which express slow MyHC only (→), slow and fast IIA MyHCs (open arrowhead), and fast IIB/IIX MyHCs only (►). This suggests that the slow, IIB/IIX and IIA MyHCs can be expressed in the absence of innervation and that innervation is not required for the expression of adult MyHCs in embryonic myotubes 28 days after injection. Bar = 55 μm for all panels except G, H and I (45 μm).

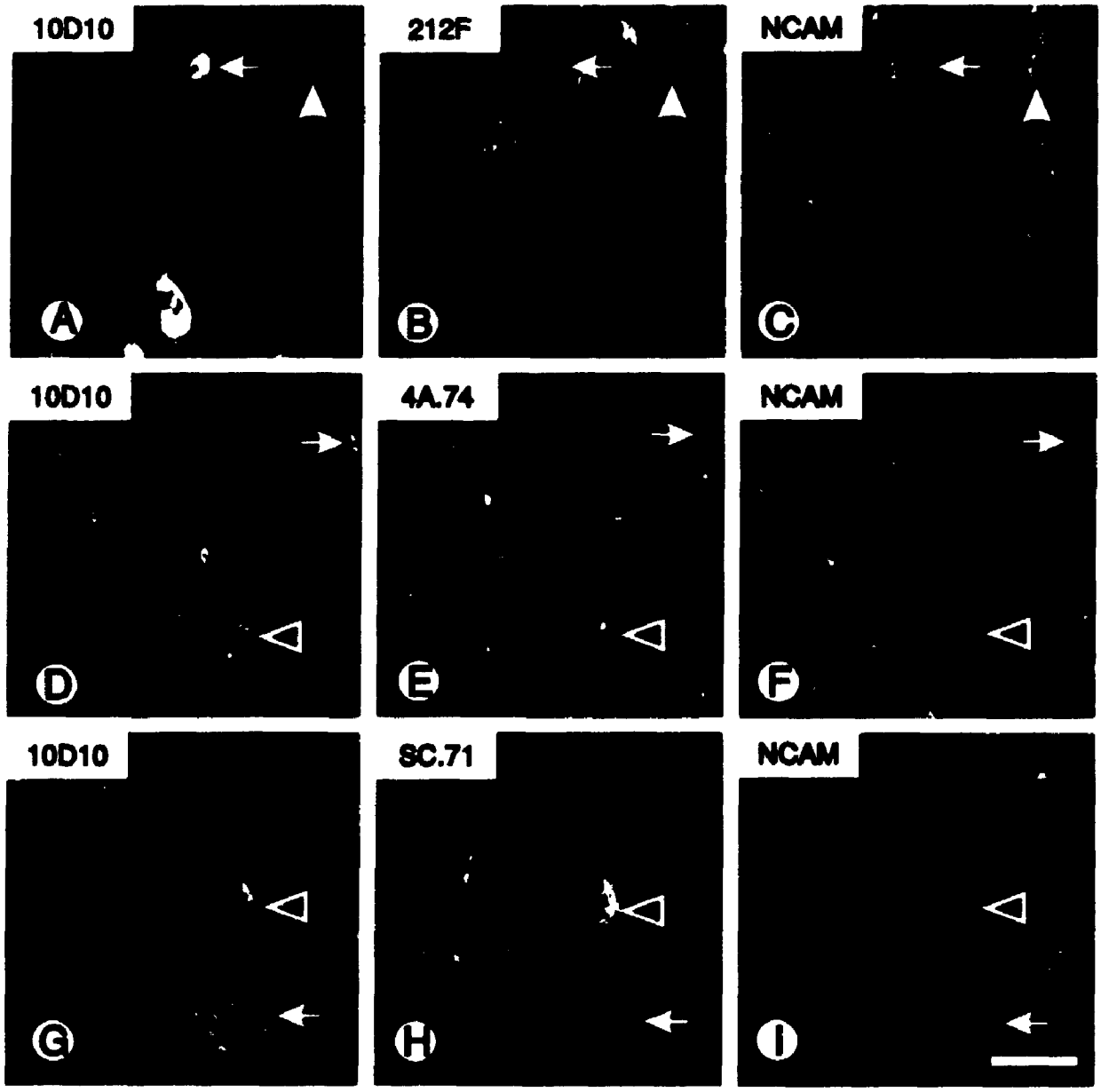
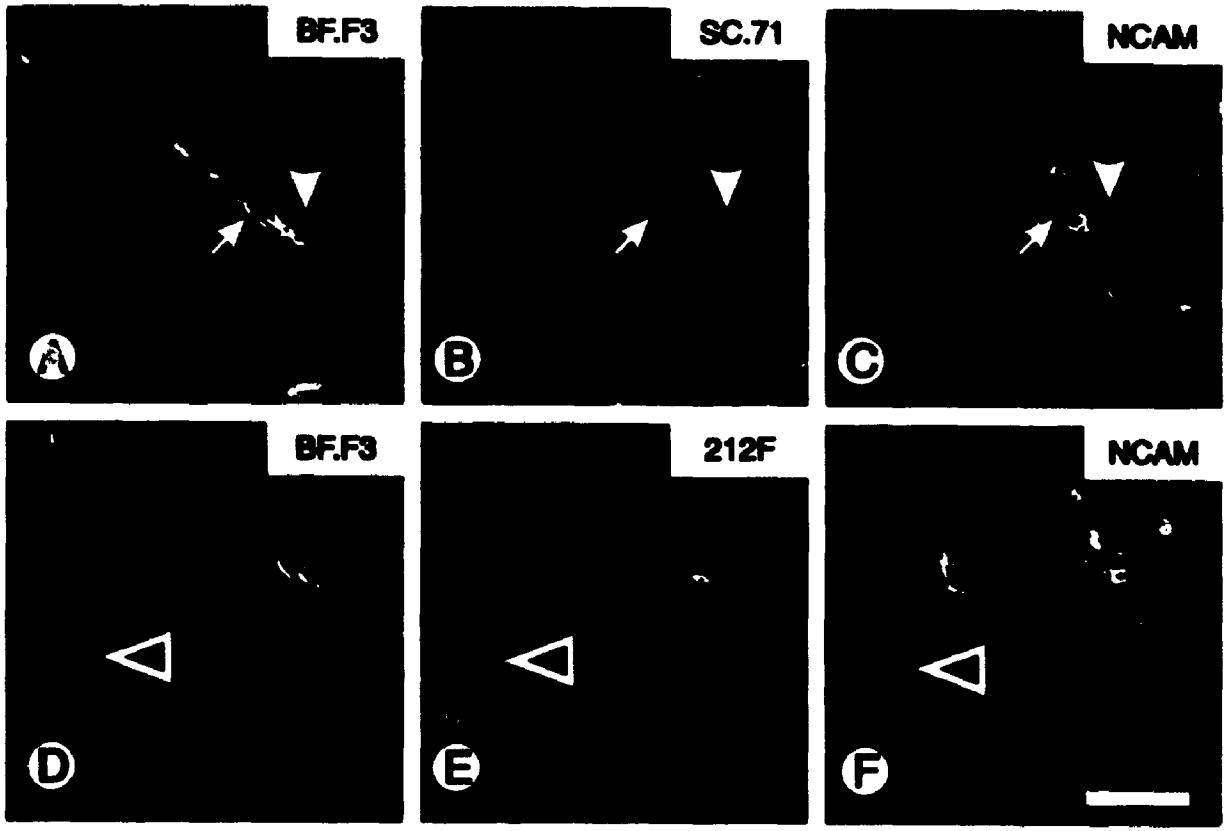

10D10
212F
NCAM
A
B
C
10D10
4A.74
NCAM
D
E
F
10D10
SC.71
NCAM
G
H
I

Figure 4.11 Confocal microscopy showing co-expression of adult fast MyHCs with NCAM in embryonic myotubes at 28 days after injection into the caudate-putamen. The same section was analyzed with antibodies specific for IIB MyHC (A;BF.F3), IIA MyHC (B;SC.71) and NCAM (C), or with antibodies specific for IIB MyHC (D;BF.F3), IIB/IIX MyHC (E;212F) and NCAM (F). Mouse Mabs against MyHCs were localized with isotype - specific secondary antibodies conjugated with either fluorescein (BF.F3) or rhodamine (212F and SC.71). The rabbit polyclonal antibody against NCAM was localized using a GAR - Cy5 conjugated secondary antibody. Triple labelling reveals NCAM positive myotubes which express IIA MyHC(▼), IIA and IIB MyHCs (→), or IIX MyHC (open arrowhead). This confirms that IIA, IIX, and IIB MyHCs can be expressed in the absence of innervation in myotubes 28 days after injection of embryonic cells Bar = 50 μm for A, B and C, 35 μm for D, E and F.


BF.F3
SC.71
NCAM
A
B
C
BF.F3
212F
NCAM
D
E
F

vation, as all of the possible myotube populations were observed to co-label for NCAM.

4.3.4 Down-Regulation of Developmental Isoforms in Both Embryonic and Fetal Myoblast Injection

The absence of 47A labelling in some myotubes suggested that embryonic MyHC is down-regulated in both fetal and embryonic cell populations. To determine the influence of innervation on the expressional pattern of embryonic MyHC expression in both fetal and embryonic cells, Mabs specific for embryonic (47A) and neonatal/adult fast (MY-32) MyHC were colocalized with the NCAM polyclonal antibody, seven, 14 and 28 days after injection (Figure 4.12). Initially, all observed myotubes in either fetal or embryonic cell populations were recognized by Mab 47A, with a subset of these also recognized by MY-32. This trend is continued at 14 days after injection since 47A positive, MY-32 negative myotubes were still observed. However, at 28 days post-injection, all observed embryonic and fetal myotubes were recognized by MY-32, while a large subset did not stain for 47A. Therefore, it appears that both embryonic and fetal cell populations down-regulated the expression of embryonic MyHC as neonatal MyHC became the predominant isoform. Analysis of the embryonic MyHC negative myotubes with the NCAM specific polyclonal antibody indicated that this transition occured in NCAM positive myotubes and, therefore, in the absence of innervation (data not shown).

To determine if the neonatal MyHC isoform can be down-regulated as well, a rabbit polyclonal antibody specific for neonatal MyHC (NN6) was colocalized with Mabs 10D10 and either MY-32 or 212F in embryonic cells 14 and 28 days after injection (Figure 4.13). Confocal microscopy of these triple localizations revealed 10D10 positive myotubes that were not recognized by either NN6 or MY-32 at both seven and 14 days after injection. This confirmed the presence of a slow MyHC only population of myotubes in these injection sites. The NN6 and MY-32 antibodies were coextensive by 14 days after injection, and at first glance, appeared to be co-

Figure 4.12 Confocal microscopy showing the co-localization of embryonic and neonatal / adult fast MyHC isoforms in embryonic and fetal cells after injection into the caudate-putamen of adult Sprague Dawely rats. Areas of brains injected with embryonic (A,C,E) or fetal (B,D,F) myoblasts were analyzed at seven (A,B), 14 (C,D) and 28 days (E,F) after injection with Mabs 47A and MY-32. Localization of 47A was detected with an FITC-RAM IgG_{2A} and MY-32 was detected with RITC-SAM IgG_1 secondary antibody. Overlap of the two fluorochromes produces a yellow colour indicating coreactivity. Seven days after injection, embryonic MyHC (recognized by 47A) is expressed either alone (green) or with neonatal / adult fast MyHC (yellow; MY-32) in both embryonic (A) and fetal (B) injection sites. Myotubes expressing only embryonic MyHC can be seen in fetal injection sites (→) and also exist in embryonic injections (not shown here). By 14 days, myotubes co-express neonatal/adult fast MyHC in most myotubes expressing embryonic MyHC (yellow) in both embryonic (C) and fetal (D) cell injection sites. At 28 days, myotubes that express neonatal/adult fast MyHC, but do not express embryonic MyHC can be detected (red, labelled with ➢) in both embryonic (E) and fetal (F) populations. Bar = 65 μm for A, 40 μm for B, 80 μm for C and D, 45 μm for E, and 25 μm for F.

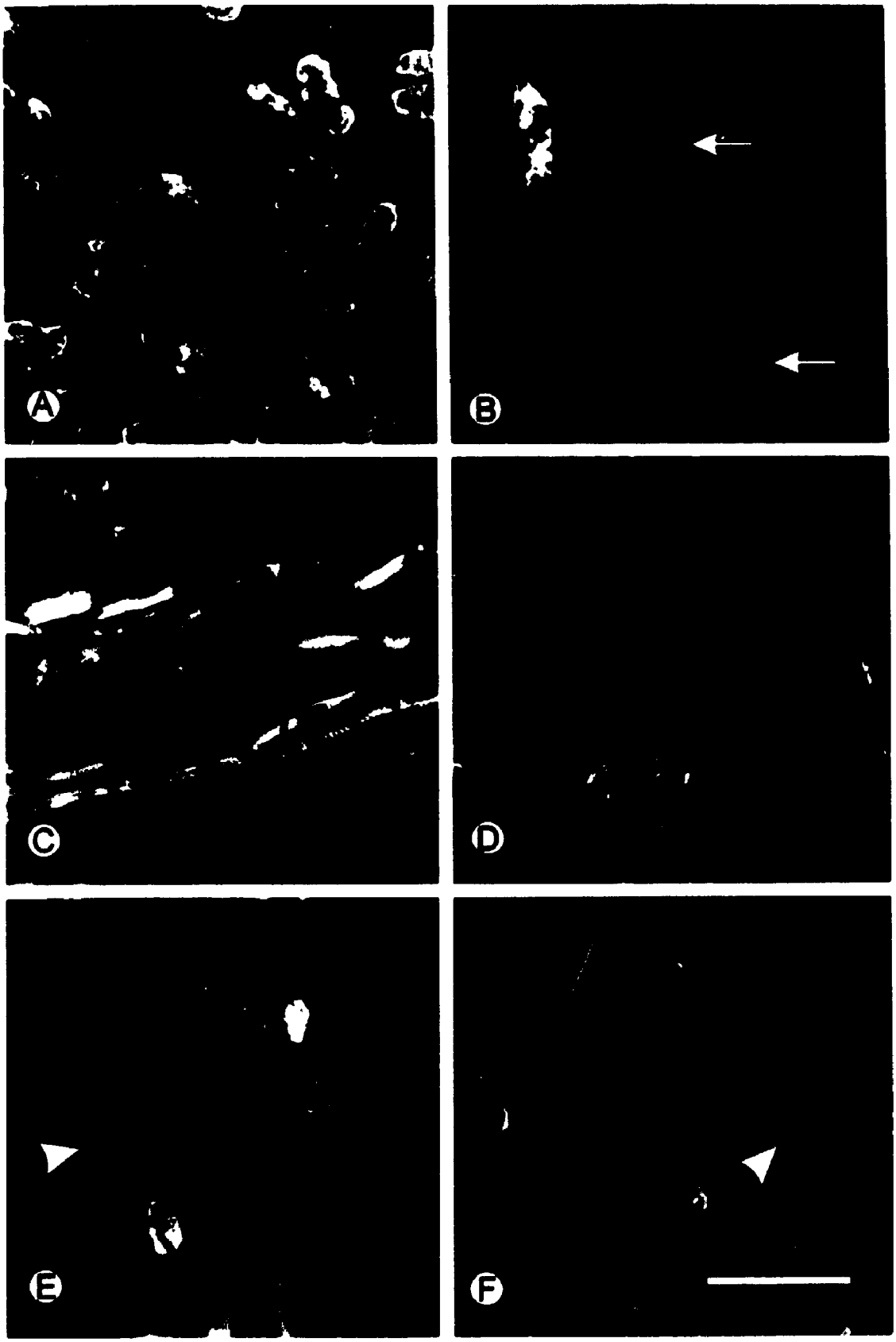
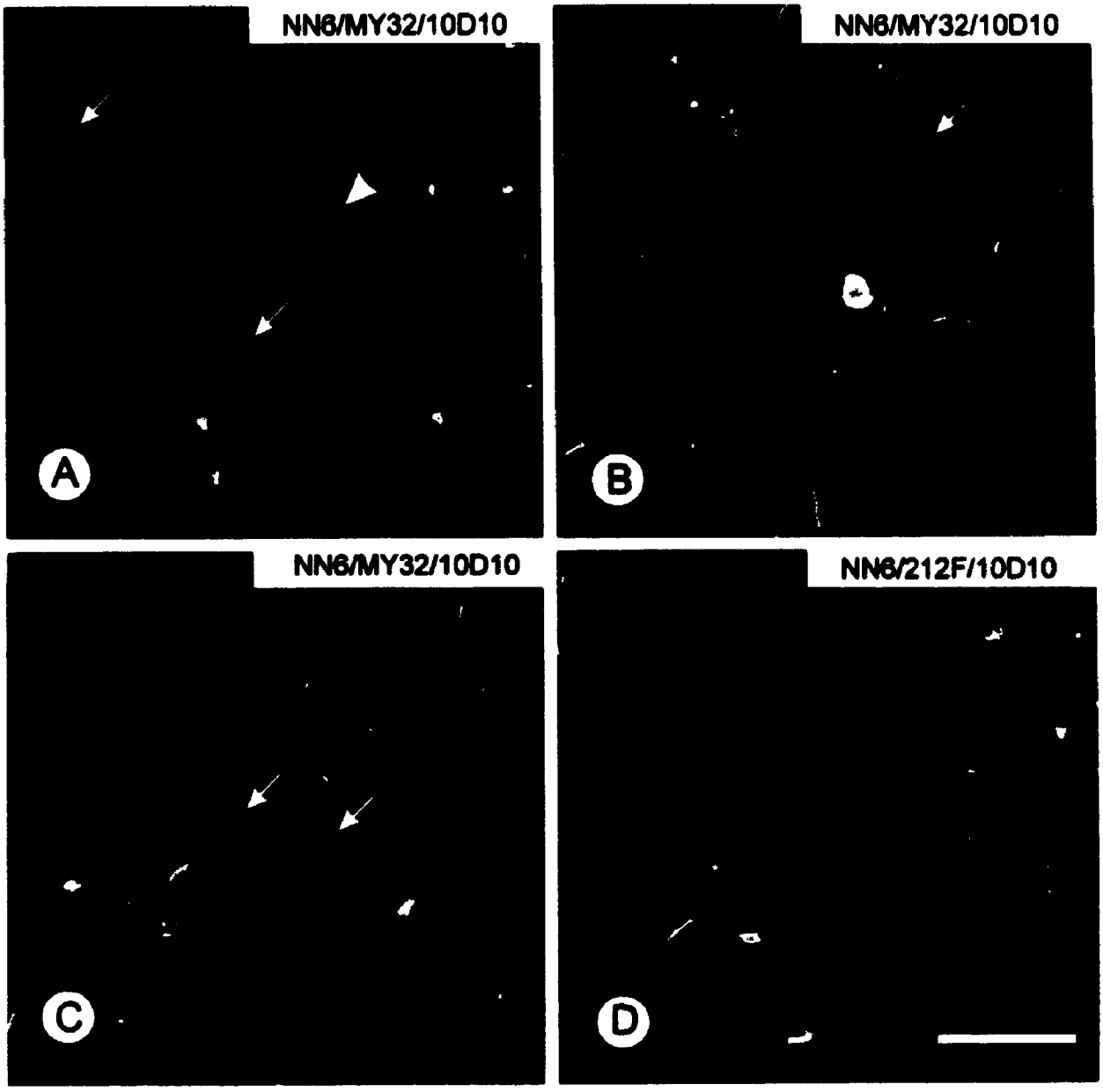

A
B
C
D
E
F

Figure 4.13 Confocal microscopy showing the co-localization of neonatal fast MyHCs with both slow and fast MyHCs in embryonic myotubes after injection into the caudate-putamen. Sections were analyzed with antibodies specific for neonatal MyHC (NN6), neonatal/adult fast MyHCs (MY-32) and slow MyHC (10D10) at 14 days (A,B) and 28 days (C) after injection, or with antibodies specific for neonatal MyHC (NN6), fast IIB/IIX MyHCs (212F) and slow MyHC (10D10) at 28 days after injection (D). FITC-RAM and RITC-SAM secondary antibodies were used to detect 10D10 or MY-32 and 212F, respectively, while NN6 was detected with a Cy5-GAR secondary antibody. Triple labelling revealed the presence of adult slow (→,green) and fast IIB/IIX (*,red) myotubes which have downregulated neonatal MyHC to attain a fully mature fibre type. Coreactivity of neonatal fast and slow MyHC was also detected (➤). Bar = 40 μm for A, 25 μm for B, 50 μm for C, and 30 μm for D.


NN6/MY32/10D10
A
NN6/MY32/10D10
B
NN6/MY32/10D10
C
NN6/212F/10D10
D

extensive at 28 days post-injection as well. However, upon closer examination of these sites, the co-labelling of NN6 with both 10D10 and 212F Mabs revealed both 10D10 and 212F positive myotubes that were not recognized by NN6. These results indicate that the neonatal MyHC can be down-regulated to produce myotubes that express only adult MyHC isoforms. Since the NN6 antibody and the NCAM - specific antibody are both rabbit polyclonals, it was impossible to determine if the down-regulation of the neonatal MyHC is nerve-dependent. However, since peripheral located nuclei were observed, it appears that fully mature muscles can be established after injection of ED 14 cells into the caudate-putamen.

4.4 Discussion

Injection of primary cells, obtained from rat embryos at ED 14, into the caudate-putamen of adult rats results in the production of graft sites histologically similar to fetal hindlimbs. Characterization of the muscle present within these grafts reveals fully mature muscle fibres with respect to their organization, state of innervation, MyHC expression and the presence of peripherally - located nuclei. Although these fibres can become innervated, no correlation was observed with MyHC expression, as all MyHC isoforms appeared prior to innervation. Myoblasts derived from ED 20 did not show as high a level of organization, but also expressed all MyHC isoforms prior to innervation. However, the presence of myotubes expressing exclusively slow MyHC within the ED 14 grafts but not the ED 20 grafts argues that these populations are different in their developmental potential. This fact suggests that there is a slow myogenic lineage present at earlier time points that does not exist later in development.

Following injection of cells derived from ED 14 (embryonic) rats, extensive proliferation and differentiation occurs, producing a graft that contains an osteogenic core, complete with endochondral ossification and a periostium, as well as large muscle fascicles surrounding this core. The appearance of all adult MyHCs indicates a developmental potential *in vivo* that is vastly different from the potential of

embryonic cells that is seen *in vitro* in rats (Chapter 2) as well as mice (Smith and Miller, 1992; Vivarelli *et al.* 1988) and chickens (Stockdale and Miller, 1987), and closely resembles myotubes found after injection of ED 20 (fetal) cells. In all, five different classes of myotubes were observed in both cell populations based on their MyHC expression; fast IIA + / slow +, fast IIA / fast IIB -, fast IIA + / fast IIB +, fast IIA - / fast IIB +, fast IIX + / fast IIB -. In addition, there is a sixth population that is exclusive to embryonic cell injections, a slow MyHC only class of myotubes. The number of myoblast classes may be an underestimation since no IIX MyHC - specific Mab is available. In the embryonic myotubes, the expression of these adult isoforms may initially be attributed to aberrant innervation since several groups have shown that innervation may be involved with the up-regulation of specific fast (Ausoni *et al.* 1990) and slow isoforms (Whalen *et al.* 1990; Gambke *et al.* 1983). However, these changes occur prior to innervation since myotubes were observed to express NCAM along their entire surface. NCAM is a surface protein that is down-regulated upon innervation, becoming specifically localized to the sub- and perisynaptic regions of the muscle fibre (Covault and Sanes, 1986). The observation that adult MyHCs appear prior to innervation agrees with other reports which suggest that both slow (Edom *et al.* 1994; Düsterhöft and Pette, 1993) and fast (Cho *et al.* 1994; Butler-Browne *et al.* 1982; Cox *et al.* 1991; Weydert *et al.* 1987) MyHCs can initially be expressed in the absence of innervation. However, these studies usually examined only a generic fast phenotype and did not address the possibility of developmental slow isoforms, which may account for a discrepancy in the literature. Therefore, this study is the first to describe the appearance of all MyHC isoforms in the absence of innervation on an individual basis.

It should also be noted that muscle fibres can be observed in which NCAM has been down-regulated indicating that innervation has occurred. Although this may affect the final phenotype of the fibres, it is unlikely that it would mimic the role of innervation found in development since this type of innervation is aberrant, probably derived from the cholinergic interneurons or even possibly from dopaminergic

neurons whose axons predominant in this area of the brain (Graybiel, 1990). It would be interesting, however, to see if the presence of the myotubes affected the phenotype of the neurons involved in the neuromuscular junctions, since previous studies have indicated a role for muscle in axon sprouting (Rutishauser and Landmesser, 1991) and motoneuron survival (Houenou *et al.* 1991).

Although direct electrical stimulation can be excluded, it is still possible that different environmental influences can exert a profound difference on MyHC expression. Since both embryonic and fetal cells exhibited a more limited level of MyHC expression *in vitro*, it seems likely that some environmental control must be exerted upon these cells *in vivo*, even if it is only to allow the myotubes to reach a higher level of maturation. Different possibilities include increases in thyroid hormone levels which have been shown to cause a shift in fibre type from slow MyHCs to progressively faster MyHCs (Izumo *et al.* 1986; Gustafson *et al.* 1986). Trophic factors derived from the spinal cord have also been shown to up-regulate the expression of fast isoforms in muscle-nerve cultures (Ecob-Prince *et al.* 1986). Since the cells in this study were injected into the caudate-putamen, both neuronal trophic factors as well as a plethora of other growth factors from the activated astrocytes were present and could have easily exerted influences on the myotube population. The production of such factors within the brain would have produced a gradient of MyHC expression as myotubes closer to the trophic source (ie the surrounding neural tissue) were more likely to be affected. However, since no such gradient was observed and myotubes within a common fascicle exhibited vastly different expressional patterns, it is unlikely that such trophic influences could account for all of the differences in phenotype. It is more likely that individual myotubes may be able to recognize separate growth factors based on their receptor expression. Differences in myoblast populations have been noted with regard to integrin expression (George-Weinstein *et al.* 1993), and it is possible that there are very subtle differences in the surface receptors which allow different populations to respond to different environmental cues. Results in cross innervation studies (Laing

and Lamb, 1983), as well as nerve-muscle cultures (Ecob-Prince *et al.* 1986) and thyroid studies (Fitzsimons *et al.* 1990; Gustafson *et al.* 1986) have shown that there is a population of fibres that are not affected by a change in environmental stimilus, suggesting that such differences may be maintained in mature muscle. However, no apparent differences described so far can account for the presence of six different myoblast populations, and it seems extremely unlikely that subtle differences could account for the appearance of all these myotube classes. Therefore, the most reasonable interpretation is that the differences observed are due in part to intrinsic controls that exist between the different populations.

Although environmental influences may be needed for the final phenotype of these myotubes to be realized, it is possible that similar results could be obtained *in vitro* if the myotubes could be characterized over longer time periods. Studies with mouse primary myoblasts, derived from later time points in gestation, revealed that the slow MyHC isoform was expressed only after 25 days in culture (Vivarelli *et al.* 1988). Alternatively, both of these scenarios could be due to a loss of myoblast programming since environmental influences may be necessary to maintain (or suppress) a particular phenotype. Evidence for this has been suggested by Cho et al (1993) who showed that the slow program of MyHC expression is gradually lost in the absence of innervation. In this study, cells were injected less than 24 hours after initial culturing so that internal programs would not be obliterated by the effects of culturing. The restricted co-expression of isoforms indicated that such a de-programming is not taking place. In fact, it appeared that the internal program was only fully recognized after several weeks *in vivo*.

The appearance of almost identical phenotypes in the two injected populations suggests two possibilities. First, it is possible that each population contains five to six different classes of myotubes. However, since *in vitro* studies have shown that the two populations exhibit markedly different patterns of expression (Chapter 2, (Smith and Miller, 1992; Vivarelli *et al.* 1988)) , which account for the entire array of phenotypes in the adult, this seems extremely unlikely. For such a scenario to

exist, two separate levels of control must be present to account for the different populations exhibited by embryonic and fetal cell cultures. A more realistic interpretation is that the embryonic cell population contains precursors for the fetal cell population. In other words, the embryonic cell population contains all of the available myoblast populations destined to form the hindlimb musculature, while the fetal cell population contains only those myoblasts still available at ED 20. This is an attractive idea since ED 14 derived cells were obtained from the hindlimb buds and adjacent back, suggesting that cells that had not migrated into the limb bud (ie. putative fetal myoblast precursors) would be included in the population of cultured cells (Seed and Hauschka, 1984). The inability of this population to differentiate in the embryonic cell cultures indicates that specific factors are present in development which allow muscle precursor cells to become myoblasts. These factors are present in the damaged brain but are absent in culture. Without the presence of such factors, cells destined to becoming myoblasts remain undifferentiated. In ED 20 hindlimb populations the cells have probably already made the transition into myoblasts and can differentiate in culture. A second observation that indicates that factors necessary for final differentiation are missing in culture, is the presence of different tissue types within the injection sites that are not found in culture (ie. cartilage and bone). Culturing of the embryonic cells in the presence of various growth factors (Cusella-De Angelis *et al.* 1994), as well as on different extra cellular matrices (George-Weinstein *et al.* 1993) has not produced any noticeable changes to the differentiation of embryonic cells. Therefore, it appears that a combination of factors may be involved in allowing the further development of myogenic precursor cells into myoblasts. If this is true, then the different myotube classes present in the embryonic cell injections would represent all of the myoblasts available for hindlimb muscle development. The slow MyHC only population of myotubes in the embryonic cell injections, therefore, represents the differentiated population of myocytes observed in culture. These cells would, therefore, represent the embryonic myoblasts that fuse to form primary myotubes *in vivo* (Duxson and Usson, 1989),

and are absent at later time points in development. The persistance of only a very small population of slow myocytes in fetal cultures (« 1%, Chapter 2) could account for the complete absence of a slow MyHC only population within the fetal cell injections.

The hypothesis that there are three general populations of myotubes - a fast, a fast/slow, and a slow, and that the slow population is limited to early in development, has previously been suggested by Stockdale and Miller for avian myogenesis (Miller *et al.* 1985). The results described in the present study actually extend these observations by identifying specific fast isoforms which can be co-localized with slow MyHC (IIA), and fast isoforms that cannot (IIB and IIX). Analysis of mRNA transcripts in mature muscle fibres has revealed the co-expression of MyHCs similar to the expression described here (DeNardi *et al.* 1993). Fast IIA transcripts were the only fast transcripts to co-localize with slow MyHC. In fact, characterization of slow muscle fibres undergoing a transition to IIX or IIB fibres revealed the sequential expression of IIA followed by IIX and then IIB (Mira *et al.* 1992). The only discrepancy between the results presented here and the mRNA characterizations was that fast IIA and fast IIB transcripts can be co-expressed in myotubes after injection into the brain. Combined, these results suggest that there is a restriction to the co-expression of MyHCs in differentiated myotubes. The fact that this restriction is established in the absence of innervation suggests that the constraints are established by intrinsic mechanisms within the myotubes themselves. Since myoblast precursors are obtained prior to their final differentiation into myotubes, it seems very likely that such a control exists prior to fusion.

It has previously been suggested that myoblast populations may be restricted in their ability to fuse to all other myoblast classes (Duxson *et al.* 1989). Observations presented in Chapter 3 suggest that this is not the case when dealing with the general populations of embryonic and fetal cells. However, since the full developmental potential of the fast populations are not seen *in vitro*, it is possible that a specific fast population may still be limited in its fusion ability. A more

attractive proposal is that myoblast classes overlap in their developmental potential. The presence of IIA MyHC expressing myotubes which colocalize with either slow or IIB/IIX MyHC supports the hypothesis that different myoblast populations represent a continuum of expression with adaptive ranges (Westgaard and Lomo, 1988). The final maturation and phenotype of a muscle fibre may be governed by extrinsic factors such as the presence of growth factors or innervation. However, this process may be restricted with specific populations of myotubes having a specific range of phenotypic expressions determined by the myoblast lineage from which they were formed. Therefore, I propose that these myoblast populations represent distinct myogenic lineages that have restrictions on the range of MyHC expression.

CHAPTER 5 - MyHC EXPRESSION OF L6 MYOBLASTS *IN VITRO*

5.1 Introduction

The analysis of MyHC expression in embryonic and fetal cell populations injected into the brain, described in the preceding chapter, suggested that development into mature muscle fibres can take place in the absence of innervation. This suggests that the development of different fibre types may be the result of intrinsic programs inherent to different myoblast populations. However, in the absence of a typical muscle environment (ie. extracellular matrix, growth factors, etc.) and appropriate innervation, it was impossible to fully evaluate the importance of environmental influences on the intrinsic myogenic programs. To evaluate the interaction between extrinsic and intrinsic influences on the development of muscle fibre types, it is necessary to transplant such a population of myoblasts into different muscles and examine the resulting phenotype of the myoblasts. Unfortunately, the existence of several different classes of myoblasts within each developmental population would tend to make interpretation of the resulting phenotypes within the muscle extremely difficult. Labelling of these cell populations with appropriate molecular markers would also require extensive culturing, which could affect the established intrinsic programs. To avoid these problems, the L6 myoblast cell line was used for a series of myoblast transplantation studies. Since the cell line was initially established from neonatal rats, one might expect that it would exhibit similar characteristics to the fetal cell population. This would make them an ideal choice for subsequent injection studies. Prior to the injection experiments, L6 cells were analyzed *in vitro* to determine their default pattern of expression.

The L6 myoblast cell line described in this study was originally derived by exposing primary cultures of neonatal rat myoblasts to a known mutagen, methylcholanthrene, *in vitro* (Yaffe, 1968). Expansion and continued culturing of a group of these myoblasts revealed the production of an immortal cell line which retained the ability to differentiate into myotubes. Previous characterization of the

L6 myotubes *in vitro* by immunohistochemistry and Northern blotting has revealed the presence of a solitary embryonic MyHC isoform (Wieczorek *et al.* 1985). Further analysis using PCR (Muthuchamy *et al.* 1992) has shown trace amounts of neonatal, adult fast IIA and slow MyHC gene transcripts. In this study, a panel of Mabs specific for developmental and adult isoforms of both fast and slow MyHCs were used to determine if these mRNAs are translated into protein during L6 myoblast differentiation *in vitro*. Both immunohistochemical localization and Western blot analysis were used to characterize MyHC expression in the L6 myoblasts during differentiation *in vitro*. These characterizations indicate that the L6 cell line expresses a very limited array of MyHC isoforms. This expression pattern is different from the MyHC profiles of several mouse cell lines, including C2C12 (Yaffe and Saxel, 1977)and Sol8 (Mulle *et al.* 1988), which express most of MyHCs in culture (MacIntyre, 1995).

5.2 Methods and Materials

Unless otherwise stated, chemicals were supplied by BDH Inc., Toronto, Ontario.

5.2.1 Culturing of L6 cells

A subclone of the L6 rat myoblast cell line originally isolated by Yaffe (1968) was obtained from Dr. B.D. Sanwal, Department of Biochemistry, University of Western Ontario.

Log phase L6 myoblasts were plated in six-well plates (Fisher Scientific, Unionville, Ont) at 8.5×10^4 and grown in complete alpha-minimal essential medium (α-MEM; Gibco/BRL, Mississauga, Ont.) containing 10% horse serum (HyClone Labs Inc, Logan, Utah) and 50 µg/ml of gentamycin (Gibco/BRL, Mississauga, Ont) as previously described (Clarke *et al.* 1989) at 37°C in an atmosphere of 4% CO_2. The media was replaced with 2 mls/well of fresh media after three and six days in culture. Under these condition, approximately 50% of cell nuclei are contained within fused myotubes after 5 days.

5.2.2 Immunolocalization of MyHC Expression in L6 Myotubes *In Vitro*

At 4, 5, 6, 7, 8 and 9 days after plating, duplicate plates were fixed in 90% methanol at -20° for 6 min and analyzed for MyHC expression using ABC immunohistochemistry as described in section 2.2.5. The panel of MyHC-specific Mabs used to characterize these cultures included Mabs specific for embryonic (Mab 47A), slow (Mabs 8H8, A4.840, 4A9, 10D10 and 4A.951), neonatal and adult fast (MY32), neonatal and adult fast IIA/IIX (Mab N1.551), adult IIB/IIX (Mab 212F), adult fast IIA (Mabs 4A.74; SC.71), adult fast IIB (Mab BF.F3), and all isoforms except IIX and embryonic (Mab BF.35) MyHCs. The specificity and optimal dilutions (in phospate buffered saline (PBS) containing 0.1% BSA (Gibco/BRL, Mississauga, Ont)) of each antibody are summarized in Table 2.1. Following immunohistochemistry, wells were coverslipped with Aquamount and photographed on a Zeiss Axiophot photomicroscope using Fujicolor 100 ASA or Kodak Tech Pan 2415 film (commercially available).

5.2.3 Western Blot Analysis of MyHC Expression in L6 Myoblasts *In Vitro*

Log phase L6 myoblasts were cultured as described for immunolocalization, except that they were plated on 100 mM culture dishes (Fisher Scientific, Unionville, Ont) at $5x10^5$ cells. At 5, 7 and 9 days of culture, myosin was isolated by extraction in high salt buffer (0.6 M NaCl, 15 mM TRIS-HCl, pH 7.4) and precipitated by dialysing against 50 vol of cold water overnight to lower the ionic strength of the extract, as described by Burridge and Bray (1975). After resuspending in 75 mM Tris and 600 mM NaCl, the protein concentrations of the myosin extracts were determined using the Lowry procedure (Lowry *et al.* 1951) and samples were diluted 4:1 with 5X Laemmli sample buffer (Laemmli, 1970) containing bromophenol blue (Biorad, Mississauga, Ont) and boiled for one min prior to electrophoresis.

SDS polyacrylamide minigels containing 8% acrylamide (Biorad, Mississauga, Ont) were prepared and run following Laemmli's protocol (Laemmli, 1970) for protein electrophoresis. 5µg of each sample was loaded per well and

electrophoresed for a total of 230 Vhrs. Samples were then blotted onto a nitrocellulose membrane (Biorad, Mississauga, Ont) at 8 W (45 V) for 20 hrs as described by Towbin et al (1979). One replica was stained with amido black and then destained in 90% methanol and 2% acetic acid to determine the success of the transfer. The other replicas were stored at 4°C in PBS until immunostaining was performed on them. Replica blots were blocked for 30 min at 37°C with 5% non - fat dry milk (NFDM) in PBS. For analysis by indirect - horseradish peroxidase (HRP), primary Mabs were diluted in 5% NFDM at the same concentration used for immunolocalization. All Mabs have been obtained from mice, and a goat-anti-mouse IgG antibody, conjugated to HRP (Biorad, Mississauga, Ont), was used at 1:1000 dilution as the 2° antibody in all cases. HRP substrate buffer (10 mM TRIS-HCl, pH 7.4; Biorad, Mississauga, Ont) containing 30 µl of 30% H_2O_2 and 2.5 mg dianisidine HCl (Sigma Chemical Co, St Louis, MO) per 100 ml was used to react with the HRP-conjugated antibody to yield a brown reaction product (Towbin *et al.* 1979). When blots were reacted a second time, a substrate consisting of 30 µg of 4-chloronapthol (Sigma Chemical Co, St Louis, MO) in 20 mls of ice cold methanol, plus 60 µl of 30% H_2O_2 in 100 ml of PBS was used as a sub trate to yield a purple reaction product. For analysis of immunoblots using ABC-AP localizations, the localization procedure was identical to that described previously for cultured cells (Section 5.2.2).

5.2.4 Characterization of Slow Mabs Using Indirect-HRP Immunohistochemistry

Hindlimb muscle was obtained from Sprague Dawely rats (Charles River, Montreal, Que) at embryonic days (ED) 14 and 20, and four days and five weeks postnatally. Tissue was frozen in melting isopentane, embedded in Tissue Tek OCT (Miles Inc., Elkhart, IN) freezing compound and serially sectioned at 10 - 15 µM on a Leitz cryostat. Sections were then fixed with 90% methanol for 6 min at -20°C, blocked for 30 min with 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont) in PBS at 37°C and then incubated with the various slow MyHC-specific antibodies for 1 hr

at RT. Following incubation in the primary antibodies for 1 hr at RT, sections were rinsed several times with PBS and incubated in a 1:1000 dilution of HRP-conjugated goat anti-mouse (GAM) IgG (Biorad, Mississauga, Ont) in BSA-PBS for 1 hr at RT. After a final rinse, sections were incubated in a 1:8 dilution of 2 x phosphate buffer containing 0.5 mg/ml diaminobenzidine (Sigma Chemical Co, St Louis, MO) and 0.006% H_2O_2 for 15 - 30 min. Sections were then washed with PBS and coverslipped using Aquamount.

5.3 Results

5.3.1 Immunolocalization of MyHC Isoforms on L6 Myotubes *In Vitro*

To determine the developmental potential of L6 myotubes *in vitro*, cultures were analyzed for MyHC expression at various time points after plating. Fusion occurred approximately four days after plating and by five days approximately 50% of the cells had fused. Embryonic MyHC (recognized by 47A) could also be detected in some myoblasts prior to fusion, suggesting that fusion is not a prerequisite for MyHC expression within these cells. When approximately 50% of myoblasts had fused (five days in culture) several antibodies that recognized isoforms other than embryonic MyHC also reacted to L6 myotubes (Figure 5.1). Along with embryonic MyHC, detectable accumulations of an adult fast MyHC was recognized by MY-32 and 212F. Since Mabs specific for IIB (BF.F3) and IIA (SC.71 and 4A.74) MyHCs did not react with the L6 myotubes, MY-32 and 212F binding most likely represents recognition of the IIX MyHC. A third MyHC isoform was also expressed in L6 cultures at five days, as 8H8 recognized several small, mononucleated myocytes and binucleated myotubes. The presence of a slow isoform was confirmed by the positive reaction of A4.840 (Table 5.1). This putative slow isoform was not recognized by all slow Mabs, however, since 10D10, 4A9, and 4A.951 showed no reactivity (Table 5.1).

At later time points in culture, the L6 myotubes expressed increased levels of IIX MyHC and proportionately less slow MyHC (Figure 5.2). While the primary

Figure 5.1 Immunolocalization of MyHC isoforms in L6 myotubes cultured for five days *in vitro*. Cultures were fixed and analyzed using ABC - AP localization of Mabs specific for embryonic (47A;A), neonatal/adult fast (MY-32;B), IIB/IIX (212F;C), IIA (4A.74;D), IIB (BF.F3; E) or slow MyHCs (8H8:F, 10D10;G, and 4A.951;H). While 47A reacts to all differentiated myotubes (A), MY-32 (B) and 212F (C) recognize only a small subset of myotubes. Since Mabs specific for IIA (4A.74;D) and IIB (BF.F3; E) MyHC do not react, 212F localization most likely represents IIX MyHC expression. Of the three antibodies that are specific for slow MyHC, only 8H8 showed a positive reaction (F). This reaction was limited to small myotubes and differentiated myocytes. This suggests that L6 myotubes express embryonic, IIX and some isoform of slow MyHC following differentiation *in vitro*. Bar = 80 μm

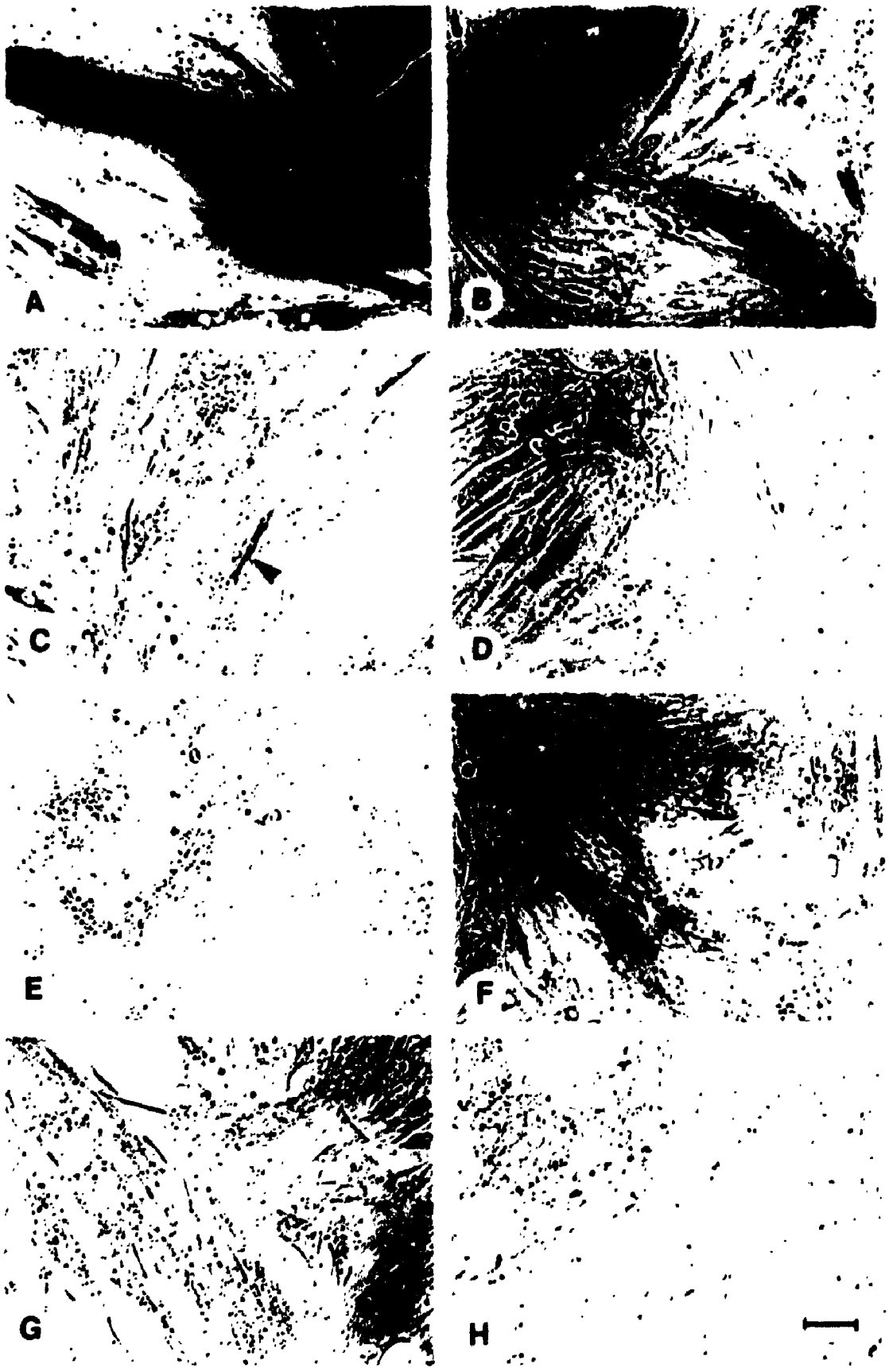

A
B
C
D
E
F
G
H

Table 5.1 Myosin heavy chain expression in L6 myotubes *in vitro* using ABC-AP Immunolocalization

Monoclonal Antibody	MyHC Specificity	Days in Culture					
		4	5	6	7	8	9
47A*	Embryonic	+++	+++	+++	+++	+++	+++
MY-32*	Neonatal, Adult Fast	-	+	++	++	++	++
N1.551*	Neonatal, IIA and IIX	-	+	+	+	+	+
212F*	IIB and IIX	-	+	+	+	+	+
4A.74*	IIA	-	-	-	-	-	-
SC.71	IIA	-	-	-	-	-	-
BF.F3	IIB	-	-	-	-	-	-
8H8*	Slow	-	+	+	+	+	+
10D10*	Slow	-	-	-	-	-	-
4A.951	Slow	-	-	-	-	-	-
4A9*	Slow	-	-	-	-	-	-
A4.840	Slow	-	+	+	+	+	+

* - can be found in figures 1 and 2
+ - recognizes less than 5% of observed myotubes
++ - recognizes less than 50% of observed myotubes
+++ - recognizes all observed myotubes

Figure 5.2 Immunolocalization of MyHC isoforms in L6 myotubes cultured for seven days *in vitro*. Cultures were fixed and analyzed using ABC - AP localization of Mabs specific for embryonic (47A;A), neonatal/adult fast (MY-32;B), IIB/IIX (212F;C), IIA (4A.74;D), IIB (BF.F3; E) or slow MyHCs (8H8:F, 10D10;G, and 4A.951;H). Once again 47A reacted to all differentiated myotubes (A), while MY-32 (B) and 212F (C) recognized only a small subset of myotubes. Since Mabs specific for IIA (4A.74;D) and IIB (BF.F3; E) MyHC still did not react, it appears that IIX MyHC is the only adult fast isoform expressed in L6 myotubes. Again, of the three slow MyHC Mabs, only 8H8 showed a positive reaction (F) which was limited to small myotubes and differentiated myocytes. This further supports the suggestion that L6 myotubes express embryonic, IIX and some isoform of slow MyHC following differentiation *in vitro*. Bar = 80 μm


A
B
C
D
E
F
G
H

MyHC isoform still appeared to be the embryonic MyHC, a greater proportion of myotubes were recognized by MY-32 and 212F. The absence of labelling for both BF.F3 and 4A 74 indicated that neither IIB nor IIA MyHC were expressed by the L6 cells, and that the adult isoform present was actually IIX MyHC. Slow expression was still observed using 8H8, but positive myotubes were both small and few in number. No labelling was observed for any other slow MyHC-specific Mab including 10D10 and 4A.951. Similar analyses which were performed at days 4, 6, 8 and 9 in culture, confirmed these observations. The expression of the various MyHC isoforms in cultured L6 myotubes is summarized in Table 5.1.

5.3.2 Western Blot Analysis of L6 Myosin Extracts

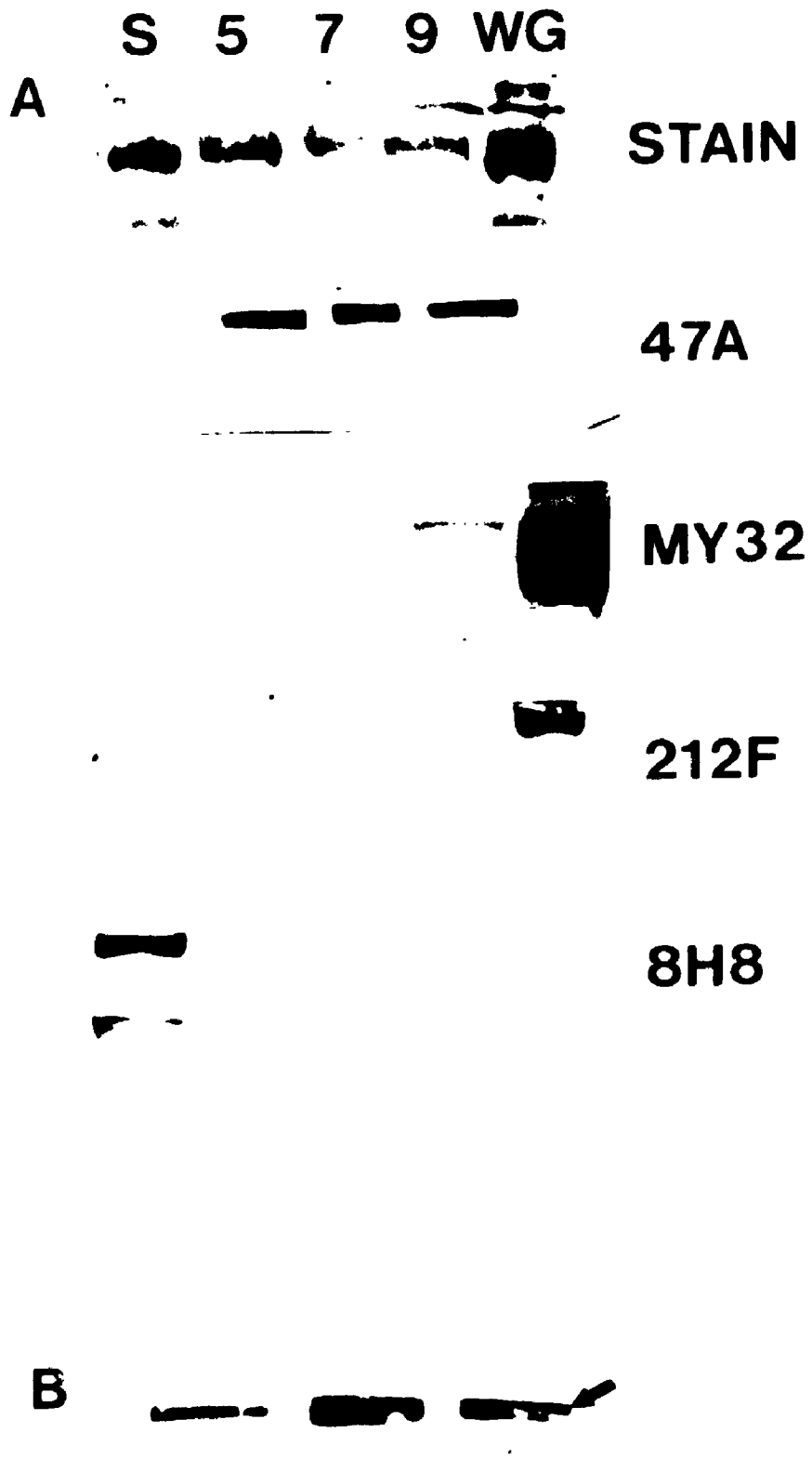
To further characterize the MyHC expression of L6 myotubes in culture, myosin extracts were obtained from cultures at five, seven and nine days after plating (Figure 5.3). Following separation on SDS - polyacrylamide gels, proteins were blotted on to nitrocellulose and Western blotting was performed using indirect - HRP immunolocalization with a variety of MyHC-specific antibodies. Amido black staining was also used to verify that equal amounts of MyHC were present for each time point. The predominating isoform detected using this assay was clearly embryonic MyHC, recognized by 47A at all time points. This antibody is highly specific for embryonic MyHC since it did not react to either the soleus (containing slow and fast IIA fibres) or white gastrocnemius (containing fast IIB and IIX fibres) muscle extracts. There was also a second isoform that was detected by both MY-32 and 212F, consistent with the expression seen in culture. It could be seen that this second isoform increased in amount over time in culture. Blotting with 8H8 did not detect the slow isoform detected in fixed cultures.

Double immunoblotting using both 212F and 47A confirms the presence of at least two distinct isoforms, ruling out 212F cross - reactivity with embryonic MyHC. This immunoblot also revealed that the isoform recognized by the 212F antibody had a slower mobility than the embryonic isoform. This migration profile

Figure 5.3 Western Blot analysis of L6 MyHC expression using indirect - HRP analysis. Myosin was isolated from L6 myoblasts cultured for five, seven or nine days, separated on 8% SDS-polyacrylamide gels, and either stained with amido black (STAIN) or analyzed using Mabs specific for embryonic (47A), neonatal/adult fast (MY-32), IIB/IIX (212F), or slow (8H8) MyHCs.

(A) Amido black analysis (STAIN) shows that equal amounts of MyHC were loaded in each lane. 47A reacts strongly with all three lanes, but does not react to MyHC isolated from the soleus (S) and white gastrocnemius (WG) muscles. Both MY-32 and 212F show an increasing reaction to the L6 myosin samples prepared from older culture as well as strong reactions to the WG sample and a very weak reaction to the S sample. 8H8 reacts with the soleus MyHC but not with MyHC isolated from L6 cultures.

(B) When MyHC samples of L6 myotubes MyHC at five, seven and nine days were run on 8% SDS-polyacrylamide gels, analyzed with both Mab 47A and 212F, and detected with either 4-chloronapthol (47A) or dianisidine (212F), two distinct bands were detected. The broad, faster migrating band, recognized by Mab 47A in all extracts, is lightly stained by the 4-chloralnaphthol reaction product. The thin, slower migrating band, recognized by Mab 212F at seven and nine day extracts, (→) is darkly stained by the dianisidine reaction product.


S 5 7 9 WG
A
STAIN
47A
MY32
212F
8H8
B

is consistent with the mobility of the IIX isoform on 8% SDS gels.

To confirm the identity of this second isoform and to increase the sensitivity of the blotting procedure, Western blots were analyzed with a larger array of antibodies using ABC-AP immunolocalization, similar to that used to detect MyHC in fixed cultures. Once again, equal amounts of myosin were separated on 8 % SDS - polyacrylamide gels and then blotted to nitrocellulose (Figure 5.4). Even with the increased sensitivity, 47A did not react with myosin samples from the gastrocnemius and soleus muscles. Mabs MY-32 and 212F once again showed increasing accumulations of a second MyHC isoform with increased time in culture. To conclusively address the identity of this second isoform, antibodies specific for adult fast IIA (4A.74) and all isoforms except embryonic and IIX (BF.35) were used. Staining with 4A.74 showed a very slight reaction to the soleus, but not to any of the L6 lanes, indicating that the second MyHC isoform is not the IIA isoform. The appearance of staining in the soleus extract is due to the small percentage of IIA fibres existing in that muscle. The BF.35 antibody reacted to both the white gastrocnemius lane (identifying IIB MyHC) and the soleus lane (indicating slow and IIA MyHCs), but it did not react to any of the L6 lanes. This confirmed that the second major isoform found in L6 myotubes was indeed the IIX MyHC.

When the 8H8 antibody was used to investigate the appearance of a slow MyHC isoform in L6 cells, a positive reaction to myosin extracts from early time points in L6 culture was observed. This band decreased in intensity over time which suggested that the slow isoform may be transiently expressed and was not present in more mature L6 myotubes. Staining with another slow antibody, 10D10, did not show a positive reaction to the L6 extracts but did react to the soleus, suggesting the existence of different slow isoforms.

5.3.3 Comparison of Slow MyHC-Specific Mabs During Development

To address the differential reactivity of the slow antibodies in L6 cultures, the specificity of each Mab was examined throughout the development of hindlimb mus-

Figure 5.4 Western blot analysis of L6 MyHC expression using ABC-AP analysis. Myosin was isolated from L6 myoblast cultured for five, seven or nine days, separated on 8% SDS-polyacrylamide gels, and either stained with amido black (STAIN) or analyzed using Mabs specific for embryonic (47A), neonatal/adult fast (MY-32), IIB/IIX (212F), IIA (4A.74) MyHCs, all isoforms except embryonic and fast IIX (BF.35) MyHCs, and slow (8H8 and 10D10) MyHCs. As expected, 47A shows strong reactivity to the L6 cultures only. 212F and MY-32 once again show increasing reactivity to the L6 extracts as time in culture increased. The absence of 4A.74 and BF.35 staining in the L6 cultures confirms that the isoform recognized by 212F and MY-32 is the adult fast IIX MyHC. The presence of 8H8 reactive MyHC is verified by bands appearing at early time points in the L6 samples, and decreasing with time. 10D10 shows no such reaction to the L6 samples, but does react with the soleus sample as expected.


S 5 7 9 WG
STAIN
47A
MY32
212F
4A·74
BF·35
8H8
10D10

culature in the rat. Three antibodies were used to carry out this analysis - 8H8, 10D10, and 4A.951. When muscle from rat fetuses at 14 and 21 days of gestation were examined (Figure 5.5), immunolocalization staining with 8H8 or 10D10 detected a large number of positively staining fibres at both time points. However, it was apparent that the 10D10 recognized only a subset of the fibres recognized by 8H8. This suggested that 8H8 recognized slow MyHCs expressed in both embryonic and fetal muscle, while 10D10 preferentially bound to a neonatal slow MyHC. Mab 4A.951, on the other hand, did not react with any of the fibres, which suggested that it did not recognize either developmental slow isoform. At four days after birth, both 10D10 and 8H8 reacted to identical populations of fibres in both the soleus and the gastrocnemius muscles (Figure 5.6). These same fibres were also recognized by 4A9.51, but at lesser intensities. By five weeks after birth, all antibodies recognized the same population of slow fibres in the soleus. Although all three slow MyHC antibodies recognized the same subset of fibres in the adult, there were obvious differences in their reactivity to developing fibres. This suggests that developmental isoforms exist during myogenesis in the rat hindlimb, similar to the developmental isoforms previously described for fast muscle fibres (Table 5.2).

5.4 Discussion

Analysis of the MyHC phenotype of L6 myotubes revealed the presence of a previously undetected adult fast isoform, IIX MyHC. The existence of IIX MyHC is supported by the reactivity of 212F and MY-32, the mobility of this isoform on SDS gels and the lack of reactivity with Mab BF.35. The appearance of only one adult MyHC suggests that the L6 myoblast population is committed to one specific adult phenotype *in vitro*, representing a default pattern of expression. The establishment of such an expressional pattern is unique since the examination of most myoblast cell lines revealed the existence of many, different, adult MyHC isoforms. The existence of such a myoblast line suggests that a preprogrammed fate can be established prior to muscle differentiation, and that this fate may determine the fibre

Figure 5.5 Indirect - HRP localization of various slow antibodies in embryonic day (ED) 14 (A,B,C) and ED 20 (D,E,F) rat hindlimbs shows a difference in the specificity of each antibody. Localization of MyHC using 8H8 (A and D) reveals binding to all of the myotubes in this field of view. 10D10 reacts to only a subset of the 8H8 - positive myotubes present at both time points (B and E), while 4A.951 does not react at all (C and F). This suggests that 8H8 recognizes both embryonic and neonatal slow MyHCs, while 10D10 preferentially binds to a neonatal slow MyHC. Mab 4A.951 does not recognize either of these isoforms expressed during fetal development. The arrows are for orientation. Bar = 50 μm


C
F
B
E
A
D

Figure 5.6 Indirect - HRP localization of various slow antibodies in the gastrocnemious (G) and soleus (S) muscles of four day (A,B,C) and five week old rats (D,E,F). Analysis with 8H8 (A and D), 10D10 (B and E) and 4A.951 (C and F) reveals that they react to similar subsets of muscle fibres at all time points after birth. Although the reactivity with 4A.951 (C) is weak at four days, it still binds to the same fibre-type populations as 8H8 (A) and 10D10 (C). This suggests that while all three Mabs react with adult slow MyHC, 8H8 and 10D10 also react with neonatal slow MyHC. The arrows denote positive fibres in the upper panel, while the stars denote negative fibres in the lower panel. Both of these symbols are meant for orientation. Bar = 80 μm

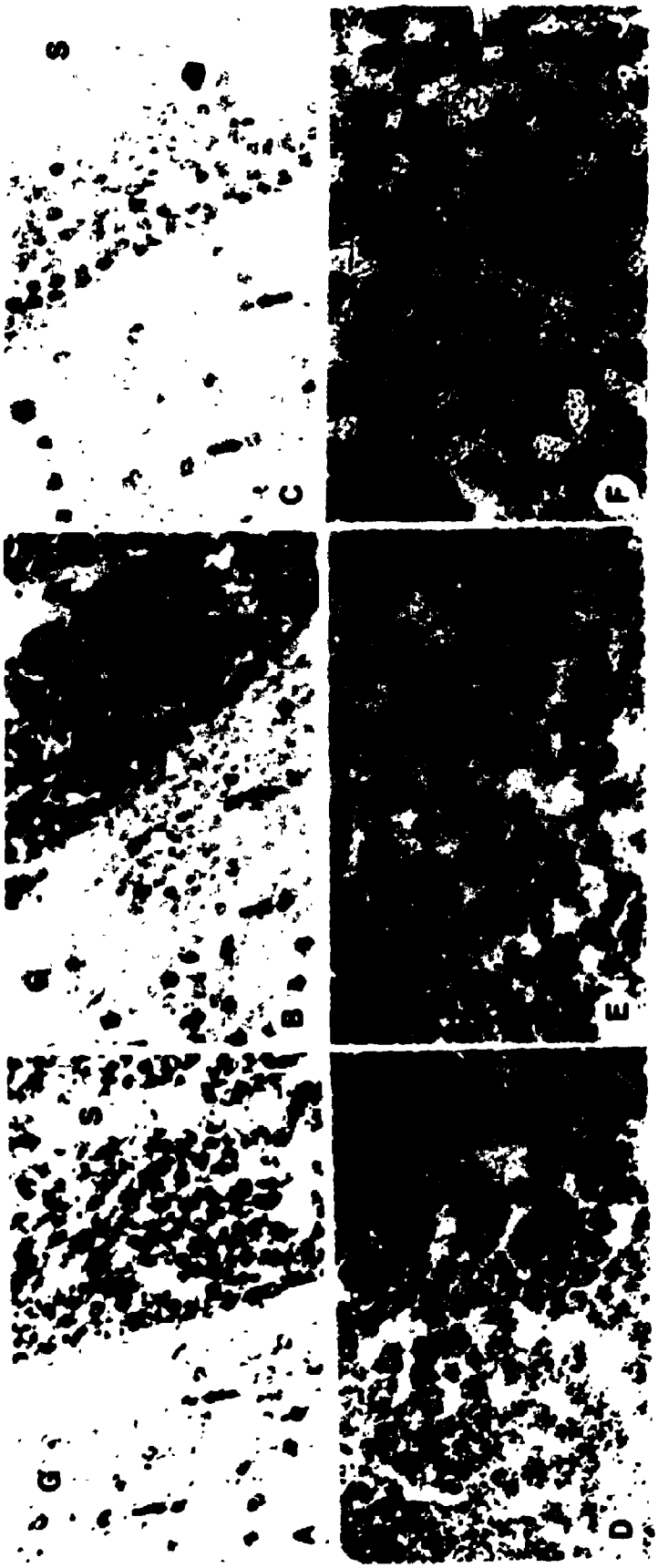

S
C
F
B
E
G
D

Table 5.2 Developmental specificity of slow myosin heavy chain specific antibodies

Monoclonal Antibody	Developmental Slow Isoforms		
	Embryonic	Neonatal	Adult
8H8	+	+	+
A4.840[a]	+	+	+
4A9	+[b]	+	+
10D10	-	+	+
4A.951[a]	-	-	+

a - as published by Hughes et al (1993a)
b - weaker than 8H8 and A4.840

type potential of the myoblast populations.

Previous studies of MyHC mRNA expression in L6 myotubes using Northern blot analysis, suggested that only embryonic mRNA transcripts were expressed throughout differentiation *in vitro* (Wieczorek *et al.* 1985). The use of reverse transcriptase driven polymerase chain reactions (RT-PCR) revealed the presence of mRNA transcripts for slow, neonatal and fast MyHCs (Muthuchamy *et al.* 1992). However, these PCR results do not contradict the findings presented here since cRNA probes and primers for IIX MyHC were not used. The recent isolation of a IIX-specific cRNA probe would permit one to corroborate the presence of IIX MyHC expression. The existence of IIX MyHC in these cultures confirmed the previous observations that innervation was not necessary for the expression of this isoform (Chapter 4), although it is possible that the relative levels of this isoform may be modulated by electrical stimulation (Ausoni *et al.* 1990).

The absence of neonatal and adult fast IIA MyHC proteins suggests that either the mRNA transcripts detected by others are not being translated or that their contribution to the phenotypic profile of the L6 myotubes is negligible. The absence of the neonatal isoform as a predominant isoform is unique to the L6 cell line, since this developmental isoform has been observed in all other mammalian myoblast cell lines (Cox *et al.* 1991; Miller, 1990). It is interesting that an adult fast MyHC is expressed in the complete absence of the neonatal isoform since, during the development of hindlimb muscles, the expression of such adult fast isoforms is usually preceded by the expression of neonatal MyHC (Condon *et al.* 1990), an result that is also observed in primary myoblast cultures (Chapter 2, (Smith and Miller, 1992; Vivarelli *et al.* 1988).

The limited expression of slow MyHC in these cells is also of some interest. Although mRNA transcripts for this isoform have been observed, only limited amounts of the protein were detected in immunolocalization assays carried out in this study. The absence of reactivity with some slow MyHC-specific Mabs indicates that the slow isoform expressed by the L6 cells probably represents a developmental slow

isoform recently described by Hughes et al (Hughes *et al.* 1993). This group showed the existence of two developmental slow isoforms in addition to the adult slow isoform. The embryonic slow MyHC first appeared upon initial differentiation of the primary myotubes, while the neonatal slow isoform appeared at slightly later time points in gestation. The antibodies used here recognized similar patterns of expression with 8H8 appearing immediately and 10D10 reacting at later times. This indicates that the 8H8 antibody is recognizing all slow isoforms, while 10D10 recognizes only the neonatal and adult slow isoforms. Characterization of the slow MyHC antibodies during development indicates that slow isoform observed in L6 myotubes is the slow embryonic MyHC. Its appearance is restricted to mononucleated myocytes, similar to the expression of slow MyHC previously described in rat fetal cell cultures (Chapter 2). The absence of slow MyHC in larger myotubes could be due to dilution by other MyHC isoforms. However, this seems unlikely since the MyHC protein usually becomes localized to small domains surrounding individual nuclei. It has also been suggested that the maintenance of the slow isoform is dependent on presence of innervation (Cho *et al.* 1993). The absence of such an environmental influence could account for the gradual disappearance of the slow MyHC in L6 cultures.

Alternatively, intrinsic factors may exist which prevent the co-expression of particular MyHC isoforms. The characterization of mature muscle in adult rats, and myotubes after injection into the brain revealed such a restriction in which IIX MyHC and slow MyHC are not co-expressed in individual myotubes (Chapter 4). In fact, the transition of a muscle fibre from slow to IIX or the reverse, proceeds through the intermediate expression of IIA MyHC first (Mira *et al.* 1992). If such a restriction exists in the L6 myoblast population, with the myotubes undergoing a slow to IIX transition, this could explain the existence of very low levels of IIA MyHC mRNA transcripts observed by Muthuchamy et al (1992). The existence of an intrinsic program is supported by the co-ordinated decrease of slow embryonic MyHC and increase of fast IIX MyHC over time, and the fact that slow embryonic

MyHC was only observed in mononucleated cells, while IIX reactivity occurred in large myotubes only. The decrease of this slow isoform in L6 cultures over time indicates that there may be a repression of slow MyHC expression that is independent of environment.

The observations presented here indicate that the L6 cell line is specifically programmed to an adult fast IIX phenotype. However, this development is incomplete in culture since embryonic MyHC continues to be expressed. The transient existence of slow embryonic MyHC may indicate that this population may also have the potential to form slow fibres under the proper circumstances. To further examine the full developmental potential of L6 myoblasts, it would be necessary to examine the fate of these cells *in vivo*.

CHAPTER 6 - DEVELOPMENTAL POTENTIAL OF L6BAG-A4 MYOBLASTS INCORPORATED INTO HOMOTYPIC FIBRES *IN VIVO*

6.1 Introduction

Adult mammalian muscle consists of several different fast fibre types (IIA, IIB and IIX) and one slow fibre type (I), all of which can be characterized by differences in their speed of contraction (Buller *et al.* 1960), resistance to fatigue (Gauthier, 1986) and pattern of MyHC expression (Armstrong and Phelps, 1984). In addition to these adult fast isoforms, there are also several developmental isoforms, including embryonic and neonatal fast MyHCs, which are not expressed in adult muscle, but are present during muscle development (Condon *et al.* 1990) and upon muscle regeneration (Whalen *et al.* 1990). There is also recent evidence that similar slow developmental isoforms are expressed in developing muscle (Hughes *et al.* 1993). The regulation of the various MyHCs, including the down-regulation of the developmental isoforms, has been studied by many different investigators and produced a variety of results (Whalen *et al.* 1984).

Cross-innervation studies in which adult slow muscles were denervated and re-innervated by fast motoneurons demonstrated a transition in the muscle phenotype from slow to fast fibre types (Buller *et al.* 1960). Therefore, it was suggested that the type of innervation received by the muscle governed the final fibre phenotype. Experiments causing changes in the electrical stimulation patterns of the muscle also illicited similar switches in MyHC expression (reviewed by Pette and Vrbova, 1992)), indicating that the changes caused by the nerve were due to the frequency of stimulation rather than specific trophic factors. However, co-culturing of spinal cord and muscle produced the up-regulation of adult fast isoforms not normally present in cultured myotubes, even in the absence of synapse formation (Ecob-Prince *et al.* 1986). This suggested that direct innervation may not be necessary for the up-regulation of the fast isoforms, and that trophic influences from the nerve may be

sufficient. Increases in thyroid hormone levels also produce changes in muscle phenotype *in vivo* (Gambke *et al.* 1983; Butler-Browne *et al.* 1984; Izumo *et al.* 1986) suggesting that other environmental influences, aside from the nerve, can change the expression pattern of MyHC isoforms in mature muscle. Combined, all of these studies suggest that the environment dictates the final phenotye of muscle fibres, and that the nerve is involved in the overall modification of that phenotype.

Recently a growing body of evidence has suggested that intrinsic factors may be also involved in dictating the final phenotype of muscle. *In vitro* data has revealed cell lines which produce all adult fast and slow isoforms without the presence of trophic or electrical stimuli from the nerve (Cox *et al.* 1991; MacIntyre, 1995). As well, examination of cultured myoblasts obtained during hindlimb development revealed the presence of different MyHC expressional patterns depending on the time of gestation in which the myoblasts were obtained. In mice (Smith and Miller, 1992; Vivarelli *et al.* 1988), quail (Miller *et al.* 1985) and rat (Chapter 2), myoblasts cultured from embryonic limb muscle consistently showed slow patterns of MyHC expression, while myoblasts cultured from later time points express fast MyHC isoforms. The analysis of myogenesis during normal muscle development has also revealed two populations of myotubes that appear at different time points in gestation (Ross *et al.* 1987), which may account for the differences of MyHC expression observed *in vitro*. Characterization of these two myotube populations supported this observation with the early population, termed primary myotubes, exhibiting a typical slow phenotype, while the later population, termed secondary myotubes, exhibiting fast patterns of MyHC expression (Condon *et al.* 1990). In fact, recent studies have detected low amounts of adult fast MyHCs in secondary myotubes almost immediately after their inception (Cho *et al.* 1994). These findings suggest that intrinsic factors may establish different patterns of expression prior to full maturation and innervation.

To determine the relative contribution of intrinsic and extrinsic factors to the development of muscle fibre type, several groups have used myoblast transplantation

to analyze the fate of myoblast populations in various muscle environments (Hughes and Blau, 1992; Morgan *et al.* 1992; DiMario *et al.* 1993). Initial experiments studying the fate of labelled myoblast populations *in vivo*, have been controversial. Injection of quail primary myoblasts or satellite cells into developing chicken hindlimbs revealed the maintenance of their *in vitro* phenotype (DiMario *et al.* 1993; DiMario and Stockdale, 1995). However, the time period examined did not allow for the analysis of long term environmental effects on the phenotype, and the myotubes were not analyzed for their state of innervation. In a different series of experiments, injection of C2C12 myoblasts or satellite cells into the muscles of adult mice resulted in patterns of MyHC expression typical of the muscle environment, suggesting that innervation ultimately controls muscle phenotype (Hughes and Blau, 1992). Unfortunately, very few fibres containing donor nuclei were observed in these studies, and all of the labelled fibres appeared to be the result of the fusion of a small number of donor myoblasts with a substantially larger number of host cells. The possibility that the donor nuclei were being overwhelmed or even "turned off" by potential *trans*-acting factors from host nuclei was not addressed by the authors. In addition, the satellite cells and C2C12 mouse myoblasts used in this study expressed multiple adult MyHC isoforms suggesting that these particular myoblasts may have a large adaptive range. Therefore, these cells may be capable of adapting to all possible phenotypes. Recent experiments indicate that this is true for satellite cells *in vitro* (Düsterhöft and Pette, 1993).

In contrast, the characterization of MyHC expression in L6 myoblasts *in vitro* indicated that this cell line may represent a myogenic lineage committed to forming fast IIX muscle fibres (Chapter 5) . However, before one can conclude that these myoblasts represent such a lineage, it is necessary to examine their developmental potential with muscle environments that normally contain fibre types other than IIX. To examine the fate of L6 myoblasts *in vivo*, these cells were injected into different muscle environments using myoblast transplantation techniques. The purpose of these experiments was to analyze the final fibre type of the myotubes and muscle

fibres containing L6-derived nuclei in order to determine the factors involved in regulating the development of muscle fibre types *in vivo*.

To determine if certain adaptive ranges are established for different myoblast populations, the L6 rat myoblast cell line was infected with a BAG retrovirus to produce a uniformly labelled population of myoblasts which were injected into regenerating muscles of adult rats. The injection of these myoblasts into regenerating adult muscles has two advantages. First, the denervation and muscle degeneration should induce axon sprouting (Dahm and Landmesser, 1988) and increase the likelihood that predominantly donor-derived (homotypic) myotubes will become innervated. The innervation of such myotubes, starting at four weeks post-injection, has been observed in experiments where large scale degeneration occurred prior to myoblast transplantation (Wernig *et al.* 1991). Secondly, with the degeneration of the muscle tissue induced by marcaine, the cells will be introduced into an area actively promoting proliferation and differentiation (Bischoff, 1986). Consequently, the cells are also likely to fuse with host myoblasts and fibres, resulting in the formation of heterotypic fibres.

The objective of this part of the study was to examine the MyHC expression in myotubes and muscle fibres containing predominantly L6 derived nuclei. By injecting labelled L6 myoblasts into various muscle environments, it should be possible to examine the effects of trophic influences in those areas. Also the establishment of predominantly donor-derived, homotypic myotubes should provide insight into the fate of these cells without the interference of putative trans-acting factors intrinsic to the host muscle. Finally, analysis of NCAM expression in these homotypic myotubes, should help elucidate the effects that innervation may have in modulating their final MyHC expression. The results obtained here indicate that homotypic L6BAG-A4 maintain their *in vitro* phenotypic profile of expressing only embryonic and IIX MyHCs. Interestingly, there is a transition in the phenotype of these myotubes in that the embryonic MyHC disappears starting at 28 days post-injection. Although this coincides with the innervation of these myotubes, no

correlation between this innervation and MyHC expression was observed.

6.2 Materials and Methods

Unless otherwise stated, chemicals were supplied by BDH Inc., Toronto, Ont.

6.2.1 Infection of L6 Rat Myoblasts with a ß-Galactosidase Reporter Gene

A subclone of the L6 rat myoblast cell line originally isolated by Yaffe (1968) was obtained from Dr. B.D. Sanwal, Department of Biochemistry, University of Western Ontario, and grown in complete alpha-minimal essential medium (α-MEM; Gibco/BRL, Burlington, Ont.) containing 10% horse serum (HyClone Labs Inc, Logan, Utah) and 50 µg/ml of gentamycin (Gibco/BRL, Burlington, Ont) as previously described (Clarke *et al.* 1989). The ecotrophic retrovirus producer cell line PSI 2 BAG α was obtained from the American Type Culture Collection (Bethesda, MA) and grown in complete D-MEM medium which contained Dulbecco's Modified Eagles Medium (D-MEM; Gibco/BRL, Burlington, Ont), 4.5 g/l glucose (Gibco/BRL, Burlington, Ont.), 10% fetal calf serum (HyClone Labs Inc, Logan, Utah), 10 mM penicillin, 5 mM streptomycin and 50 µg/ml fungizone (psf; Gibco/BRL, Burlington, Ont). Viruses (BAG) produced by this line contain the lacZ gene under control of the LTR promoter and the transposon Tn5 neomycin-resistance gene (neo^R) under control of the SV40 early promoter (Price *et al.* 1987). To produce transducing BAG virus, PSI 2 BAG α cells were grown for 1 day post-confluence in T75 flasks, refed with complete D-MEM medium and incubated for another 48 hr. Conditioned medium containing the BAG retrovirus was harvested, centrifuged at 800 g x 10 min and filtered through a 0.45 µM filter to remove cells and stored as 10 ml sterile aliquots at -80°C.

To infect L6 myoblasts, log phase cells (10^6/T75 flask; Fisher Scientific, Unionville, Ont) were incubated with 15 mls of complete α-MEM medium containing 4 µg/ml Polybrene (hexadimethrine bromide; Aldrich Chemicals, Milwaukee, WI) and 10% conditioned BAG cell supernatant for three 8 hr intervals, with fresh virus applied at the beginning of each interval. After infection, myoblasts

were refed with fresh α-MEM medium and allowed to recover for at least 24 hrs before being passaged. After passage, infected cells were grown in α-MEM medium containing 2 mg/ml of the neomycin analog G418 Sulfate (Geneticin; Gibco/BRL, Mississauga, Ont) for 2 weeks to kill uninfected cells, then passaged and grown at clonal density in regular medium. To assay for ß-galactosidase activity, cultures were fixed with 2% formaldehyde, 0.4% glutaraldehyde in phosphate buffered saline (PBS) and incubated overnight in X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside; Gibco/BRL, Burlington, Ont) substrate containing 1 mM $MgCl_2$, 10 mM potassium ferricyanide (Fisher Scientific, Unionville, Ont), 200 µg/ml X-gal, 150 mM NaCl and 0.1 M sodium phosphate (Fisher Scientific, Unionville, Ont) pH 7.5 for 18 hr at 37°C, as described by Shimohama et al (1989). Clones were physically isolated using cloning cylinders, expanded and duplicate wells tested for ß-galactosidase (β-gal) activity. Clones in which all cells expressed high levels of ß-gal (such as L6BAG-A4) were tested for their ability to fuse normally then expanded for injection into regenerating adult rat muscles or for freezing in complete media containing 10% DMSO (Fisher Scientific, Unionville, Ont).

6.2.2 Injection of L6 Myoblasts into Regenerating Adult Muscles

Once stable clones of L6 myoblasts were obtained in which a high, constituitive level of ß-gal could be observed, cells were expanded to obtain large populations for injection. One such clone, L6BAG-A4 myoblasts, was grown to approximately 50% confluence on 100 mm culture dishes (Canlab Scientific Products, Mississauga, Ont), rinsed once with Ca^{++}, Mg^{++} Free Hanks Balanced Salt Solution (CMF-HBSS; Gibco/BRL, Burlington, Ont), then trypsinized with a 1 in 10 dilution of 2.5% trypsin (Gibco/BRL, Burlington, Ont.) in CMF-HBSS until all of the cells lifted off the plate. The harvested cells were then collected and washed twice with CMF-HBSS. The resulting pellet was resuspended in a marcain cocktail containing 0.5 % marcain (bupivacaine hydrochloride; Sterling-Winthrop, Markham, Ont.), 30 µg/ml (30 units/ml) hyaluronidase (Cooper Biomedical) and 0.03% India

ink (Pelikan; Hannover, Germany), at a concentration of 1.0 x 10^6 cells/50 µl. Twenty-seven 2-3 month old rats were anaesthetized using a combination of sodium pentobarbital (M.T.C. Pharmaceuticals, Cambridge, Ont.) and chloral hydrate. Their hindlimbs were then shaved and swabbed in alcohol prior to injection.

Approximately one million cells in 50 µl of the cocktail were injected into each of three sites - (a) the soleus and plantaris, (b) the gastrocnemius, and (c) the tibialis anterior and extensor digitorum longus muscles of the right leg. Control injections, containing the marcain cocktail but without cells, were performed in the same manner on the left legs. A 1 ml syringe (Terumo, Elkton, Md.) with a 26-gauge needle (Terumo, Elkton, Md.) was used to perform the injections. The animals were allowed to recover under heat lamps and then maintained as an isolated colony. Each rat initially received a daily injection of cyclosporin A (Sandoz Canada Inc., Montreal, Que) at a concentration of 15 mg/kg starting body weight. After 4 weeks, cyclosporin injections were reduced to 6 out of 7 days to reduce trauma on the rat. Six rats were sacrificed at 7, 14 and 28 days, 4 rats were sacrificed at 42 and 56 days, and one rat was sacrificed at 51 days after injection. Injection sites identified by India Ink were then frozen in melting isopentane, embedded in Tissue Tek OCT (Miles Inc., Elkhart, In.) freezing compound and serial sectioned at 10 - 15 µM on a Leitz cryostat. Every tenth section was fixed in 2% formaldehyde, 0.4% glutaraldehyde in PBS and analyzed for ß-gal expression using X-gal substrate as previously described for cultured cells.

6.2.3 ABC - Immunohistochemical Analysis of MYHC Expression in L6BAG-A4-Derived Myotubes

Serial sections adjacent to areas of X-gal staining were analyzed using ABC-AP immunohistochemistry as previously described in Section 2.2.5. Sections were chosen based on the presence of peripherally located myotubes that stained darkly for X-gal, and appeared to be separated from the main muscle mass. Primary monoclonal antibodies that were used in the characterization of these myotubes

included 47A, MY-32, 4A.74, 212F, BF.F3, SC.71, BF.35, 4A9, 10D10, 8H8, and 4A.951. The optimal dilution, specificity, isotype, and source of these antibodies has previously been discussed (see Table 2.1).

6.2.4 Immunofluorescent co-localization of NCAM and MyHC isoforms in L6BAG-A4 Myotubes

Serial sections adjacent to areas of X-gal staining were analyzed for the co-expression of NCAM with MyHC isoforms. Sections were chosen in the same manner as described in 6.2.3, and then blocked in 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont) in PBS for 30 min at 37°C and incubated in primary antibody. Since the NCAM antibody is a rabbit polyclonal antibody, and the MyHC specific antibodies are mouse monoclonal antibodies, both primary antibodies were incubated simultaneously. Primary Mabs used included 47A (1:10), MY-32 (1:100), 8H8 (1:40), and BF.35 (undiluted). Following incubation in the primary antibodies for 45 mins at RT, sections were rinsed several times with PBS and incubated in fluoroscein-conjugated goat anti-rabbit (FITC-GAR) IgG and rhodamine-conjugated goat anti-mouse (RITC-GAM) IgG secondary antibodies (both supplied by Tago Inc, Burlingame, CA), both diluted to 1:50 in BSA-PBS. The cultures were then washed several times with PBS and coverslipped with a mountant containing 50% glycerol and 5% paraphenylene diamine. Hoescht dye 33252 (Sigma Chemical Co, St Louis, MO) was added at a concentration of 0.5% to uniformly label all nuclei within the culture. The fluorescence labelling was photographed on Kodak T-Max 400 (black and white) or Fujicolour 400 ASA (colour) films (commercially available) using a Zeiss Axiophot photomicroscope equipped with a filter set 17 for FITC (exciter filter BP485, barrier filter 515-565) and filter set 15 for TRITC (exciter filter BP 546, barrier filter BP 590) using a 40X Neofluar objective.

6.3 Results

6.3.1 Characterization of MyHC expression in regenerating adult rat muscle fibres following injection of marcain and hyaluronidase

Following injection of 0.5% marcain containing hyaluronidase, adult muscle undergoes widespread necrosis in which individual muscle fibres degenerate. This degeneration is followed closely by regeneration of the fibre through repair by satellite cells. Initially, the characterization of these regenerating fibres using ABC-AP immunohistochemistry with MyHC - specific antibodies was carried out in the plantaris, gastrocnemius, and tibilais anterior muscles at various times after marcaine injection.

Characterization of a regenerating plantaris muscle seven days after marcain injection (Figure 6.1) showed an extensive area of necrosis, in which small myotubes were separated by relatively large gaps of connective tissue. These myotubes showed a characteristic pattern of central nucleation (not shown here) and had a smaller diameter than non-regenerating fibres. Following ABC-AP immunolocalization with various Mabs specific for different isoforms, this area was recognized by 47A, MY-32 and BF.35, all of which recognize developmental isoforms. A positive reaction in many of the regenerating myotubes was also observed for 212F indicating the presence of an adult fast isoform. The fast isoform that was expressed in the regenerating area was probably IIX, since a negative reaction was seen for both BF.F3 and 4A.74, which react to the IIB and IIA isoforms respectively. Mabs recognizing the various slow isoforms did not react with the regenerating myotubes, suggesting that the expression of the slow isoforms was a later event in plantaris muscle regeneration. Therefore it appears that the initial pattern of MyHC expression in regenerating fibres is limited to embryonic, neonatal and fast IIX MyHCs.

By 28 days post-injection, muscle histology was normal at the injection sites with polygonal shaped fibres separated by very little connective tissue (Figure 6.2).

Figure 6.1 Pattern of MyHC isoform expression in the regenerating plantaris muscle of adult rats seven days after marcain injection. MyHC expression was examined using ABC-AP immunolocalization with Mabs specific for embryonic (A;47A), neonatal/adult fast (B;MY-32), all isoforms except IIX and embryonic MyHCs (C;BF.35), fast IIB/IIX (D;212F), fast IIB (E;BF.F3), fast IIA (F;4A.74), all slow (G;8H8) and neonatal/adult slow (H;4A9), and adult slow (I;4A.951) MyHCs. A zone of regeneration, initially identified by India ink particles, can be seen in the upper part of the panels. Mabs MY-32 (B), BF-35 (C) and 212F (D) also react with these fibres. Since they do not express IIB MyHC (BF.F3; E), IIA MyHC (4A.74;F), and slow MyHC (G,H and I), MY-32 and BF.35 are probably reacting to neonatal fast MyHC in these regenerating fibres. Reaction with 212F probably represents IIX MyHC expression. Bar = 180 μm.

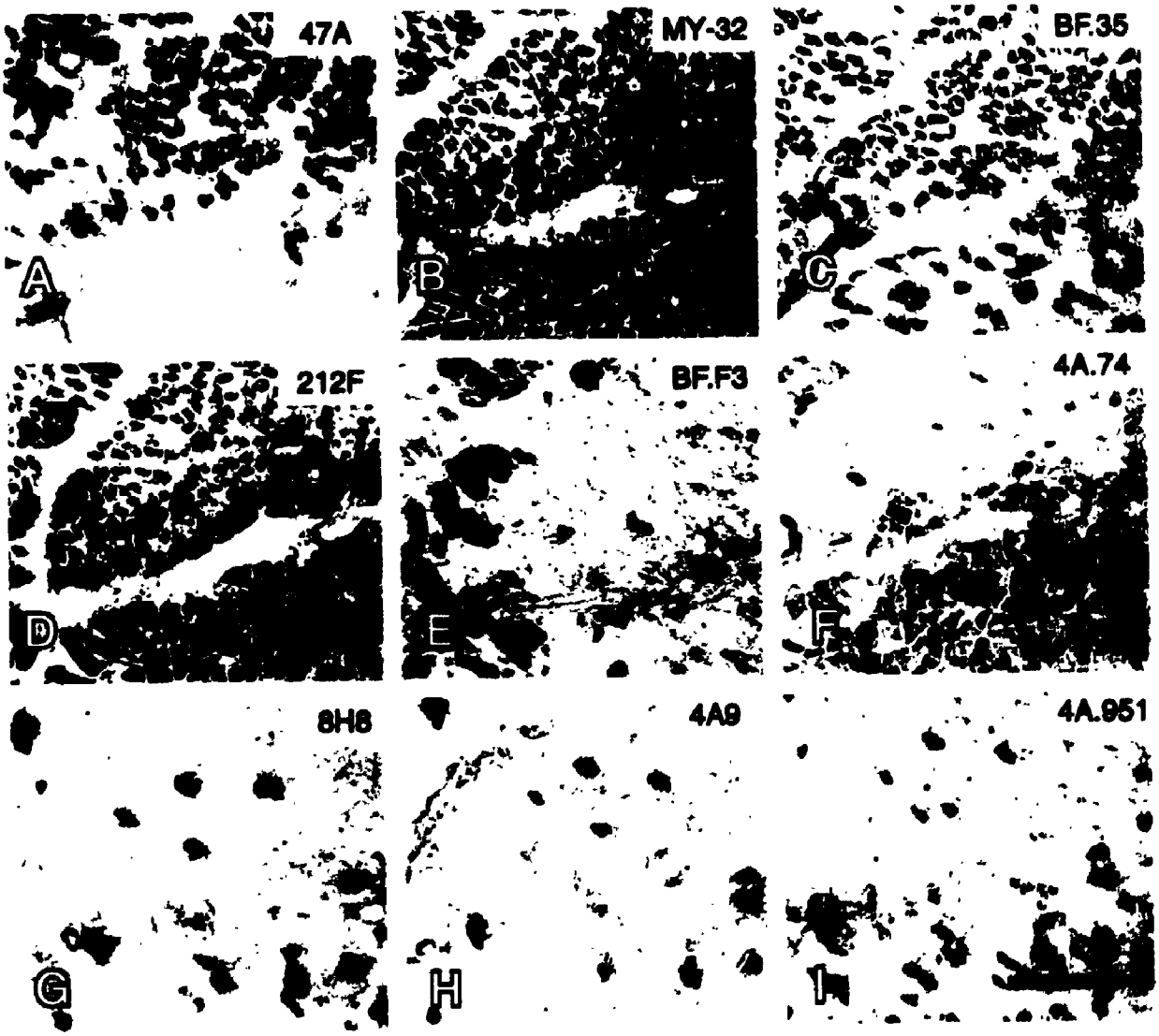
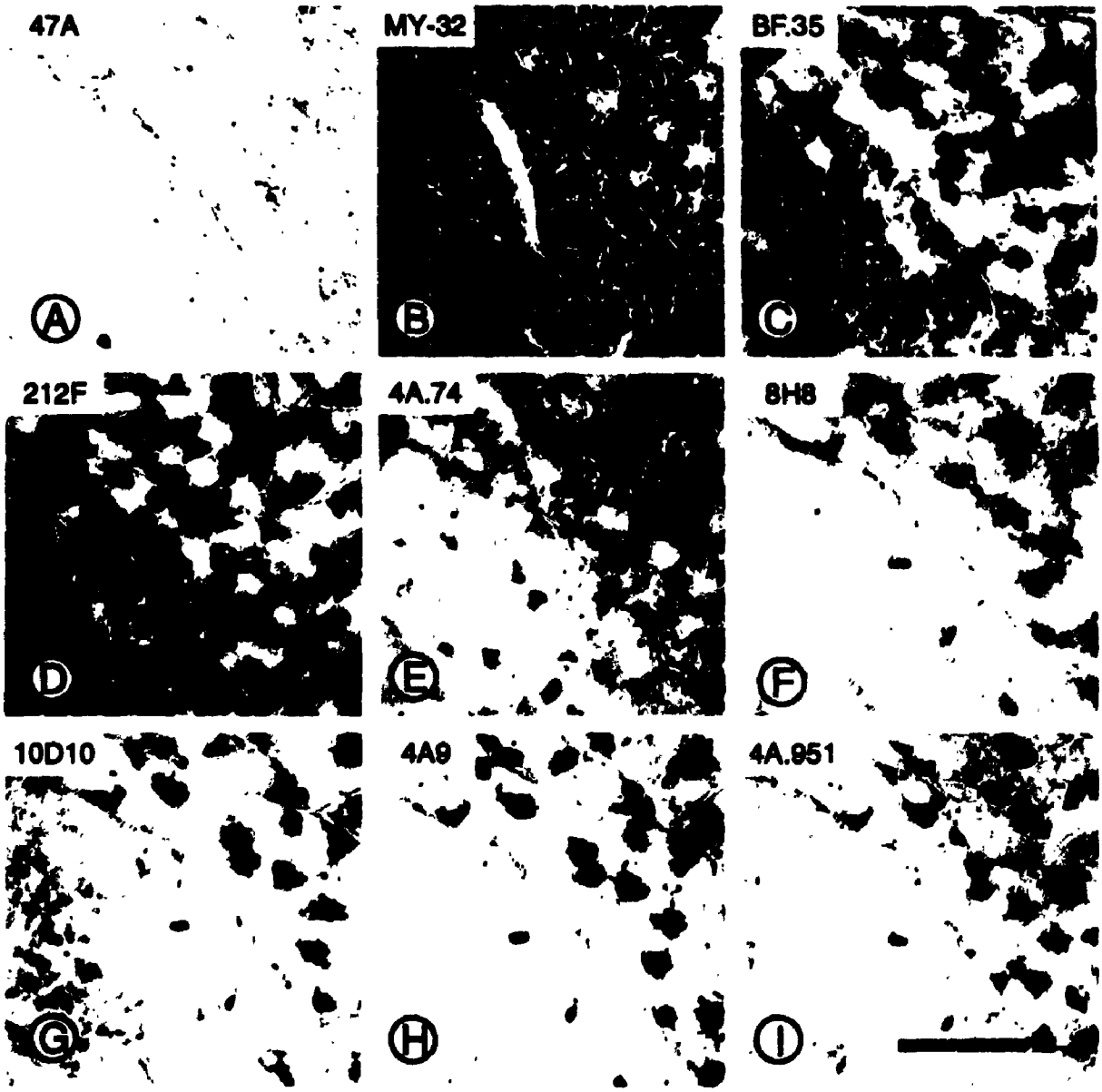

47A
A
MY-32
B
BF.35
C
212F
D
BF.F3
E
4A.74
F
8H8
G
4A9
H
4A.951
I

Figure 6.2 Pattern of MyHC isoform expression in the regenerating plantaris muscle of adult rats 28 days after marcain injection. MyHC expression was examined using ABC-AP immunolocalization with Mabs specific for embryonic (A;47A), neonatal/adult fast (B;MY-32), all isoforms except embryonic and IIX (C;BF.35), fast IIB/IIX (D;212F), fast IIA (E;4A.74), all slow (F;8H8), neonatal/adult slow (C,10D10 and H;4A9), and adult slow (I;4A.951) MyHCs. Embryonic MyHC is no longer expressed in the area that had undergone regeneration (panel A), which was identified by the presence of India Ink between the fibres. From analysis with MY-32 against neonatal/adult fast MyHC and slow specific Mabs 8H8, 10D10, 4A9 and 4A95, suggest that the regenerated portion of the plantaris muscle contains both fast and slow fibres. Reactivity with Mabs 212F, 4A.74 and BF.F3 suggest that mature IIA, IIB and IIC fibres are all present. All slow staining fibres (→) show reactivity to all slow antibodies (E-H) indicating that they have also fully matured. This suggests that a normal pattern of adult muscle fibre types is established in the plantaris by 28 days after injection with marcain. Bar = 255 μm.


47A
MY-32
BF.35
212F
4A.74
8H8
10D10
4A9
4A.951
A
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Upon initial observation, the only indication that the area had undergone degeneration was the presence of India ink particles located between the fibres. Further observation with nuclear stains revealed centrally located nuclei (not shown here), which are also a characteristic of regenerated fibres. Upon ABC-AP immunolocalization with the panel of MyHC specific antibodies, 47A did not react to any of the muscle fibres indicating that embryonic MyHC was no longer expressed in this muscle. Although MY-32 showed extensive reaction to many of the fibres, this was colocalized with either 212F or 4A.74, suggesting that these were mature adult fibres. All of the Mabs against slow MyHCs recognized the same subset of fibres, which did not react with the fast antibodies. This indicated that the slow fibres undergoing regeneration reached maturity by 28 days after injection and expressed adult slow MyHC. Reaction with BF.35, which recognizes all adult muscle fibres with the exception of IIX fibres, suggested that the fully regenerated plantaris contained many IIX fibres, since all BF.35 negative fibres were 212F positive. These fibres also showed a weak reaction to 4A.74, which has been shown to cross react to IIX MyHC when this isoform is present in high amounts. Together, this suggested that the regenerated plantaris muscle contained a mixture of I, IIA, IIB and IIX fibres similar to the normal adult plantaris muscle.

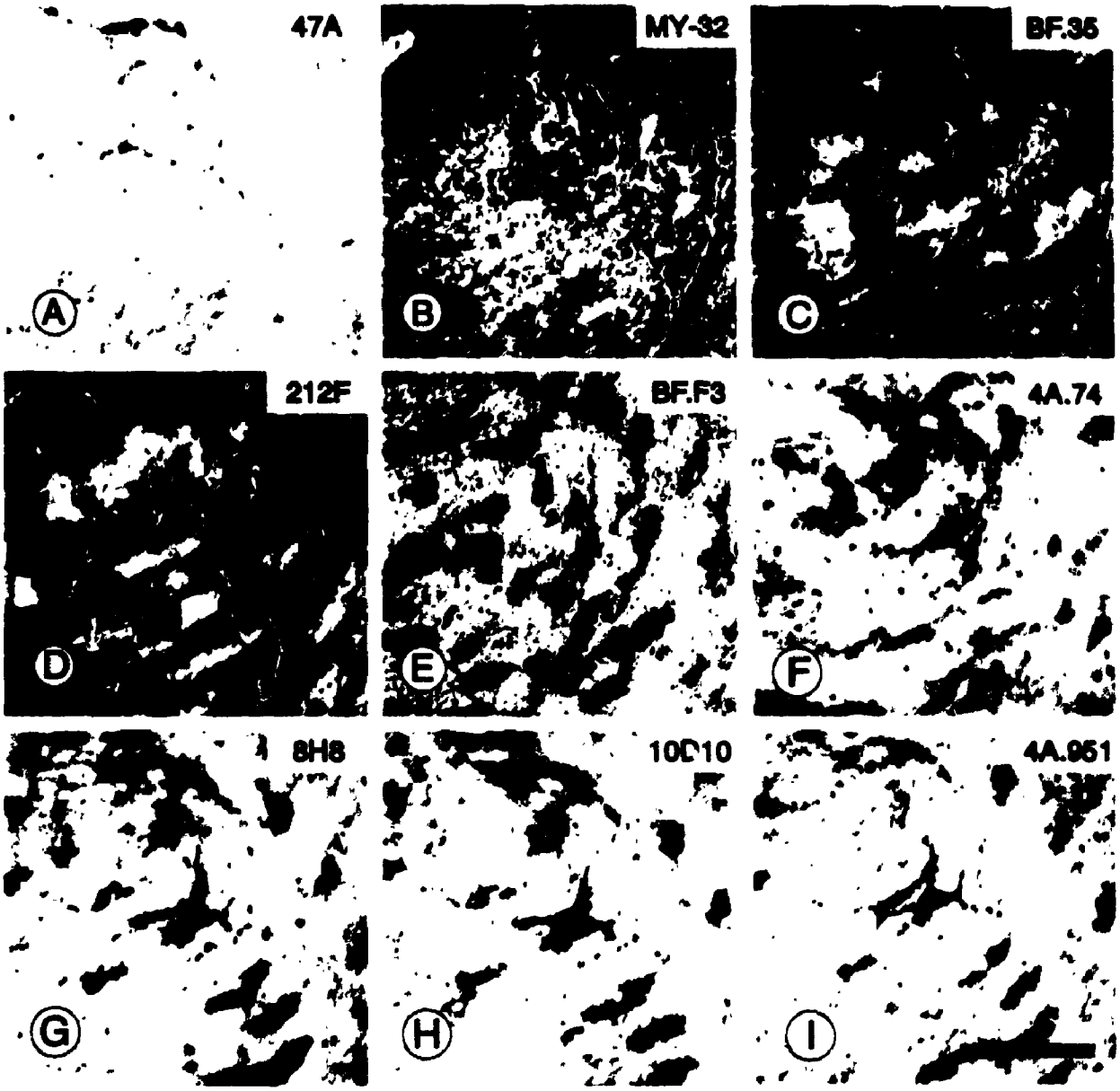
To determine if the re-establishment of normal muscle fibre type was consistent throughout all muscles, similar ABC-AP immunolocalizations were performed on the gastrocnemius, tibialis anterior and soleus muscles. Results obtained with the gastrocnemius muscle were similar to those seen in the plantaris muscle. This indicated that mature muscle fibres were present at 28 days after marcain injection into the gastrocnemius (Figure 6.3). Once again Mab 47A did not react with an area that shows India ink particles between the fibres, while 212F, BF.F3 and 4A.74 all reacted with subsets of MY-32 staining fibres. All BF.35 negative fibres reacted positively with 212F, which indicated that they were most likely fast IIX fibres. Once again, all slow fibres were stained positively by 8H8, 10D10, and 4A.951, indicating that the adult slow MyHC isoform was present.

However, there was the appearance of an occassional fibre that stained positively for both MY-32 and the slow specific Mabs. This may represent the persistence of neonatal fast MyHC or a IIC fibre, which was undergoing transition between slow and fast phenotypes. Characterizations of the other muscles at the same time point gave similar results with a very small proportion of fibres staining for both fast and slow MyHCs (data not shown).

To further document the observation that a normal fibre type distribution was obtained after marcain injection and cyclosporin treatment, a third muscle, the tibialis anterior, was analyzed 56 days after injection (Figure 6.4). There were no signs of degeneration having taken place with the exception of India ink particles between the muscle fibres. ABC-AP immunolocalization of the various MyHCs using specific Mabs revealed a normal muscle phenotype. Mab 47A did not react with any fibres which indicated that the fibres attained an adult phenotype even during immunosuppression. Once again 212F, BF.F3 and 4A.74 stained complimentary subsets of the population of muscle fibres detected by MY-32. All of the fibres not stained by MY-32 showed a positive reaction to 8H8, 10D10, and 4A.951. Fibres which did not react with BF.35 were probably IIX fibres.

Characterization of the MyHC profile of the various muscles following marcain injection indicated that there was an initial expression of developmental isoforms in the myotubes undergoing regeneration. The expression of these developmental isoforms was transient, with very few fibres expressing embryonic MyHC at 14 days after marcain injection (data not shown), and a complete absence of this isoform at 28 days post-injection. Neonatal MyHC was virtually absent by 28 days after injection, with all fibres exhibiting a mature phenotype. Suppression of the immune system did not appear to affect the normal phenotype of the various muscles 28 or more days after marcain injection, since the observed staining pattern was typical of normal adult muscle with the fibres expressing one predominant MyHC isoform (D'Albis *et al.* 1988).

Figure 6.3 Pattern of MyHC expression in the regenerating gastrocnemius 28 days after injection with marcain. MyHC expression was determined using ABC-AP immunolocalization with Mabs specific for embryonic (A;47A), neonatal/adult fast (B;MY-32), all isoforms except embryonic and IIX (C;BF,35), fast IIB/IIX (D;212F), fast IIB (E;BF.F3), fast IIA (F;4A.74), all slow (G;8H8), neonatal/adult slow (H;10D10), and adult slow (I;4A.951) MyHCs. Embryonic MyHC (A) is no longer expressed in the area that had undergone regeneration (as evidenced by the presence of India Ink between the fibres). Neonatal and adult fast isoforms are detected by staining for MY-32 (B), and all of these fibres stain for either fast IIB/IIX (D), fast IIB (E) or fast IIA (F), suggesting that they are mature. All slow staining fibres (→) show reactivity to all slow antibodies (G,H,I) indicating that they have fully matured. This suggests that normal adult muscle fibre type is established in the gastrocnemius by 28 days after injection with marcain. Bar = 215 μm.


47A
MY-32
BF.35
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212F
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4A.74
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E
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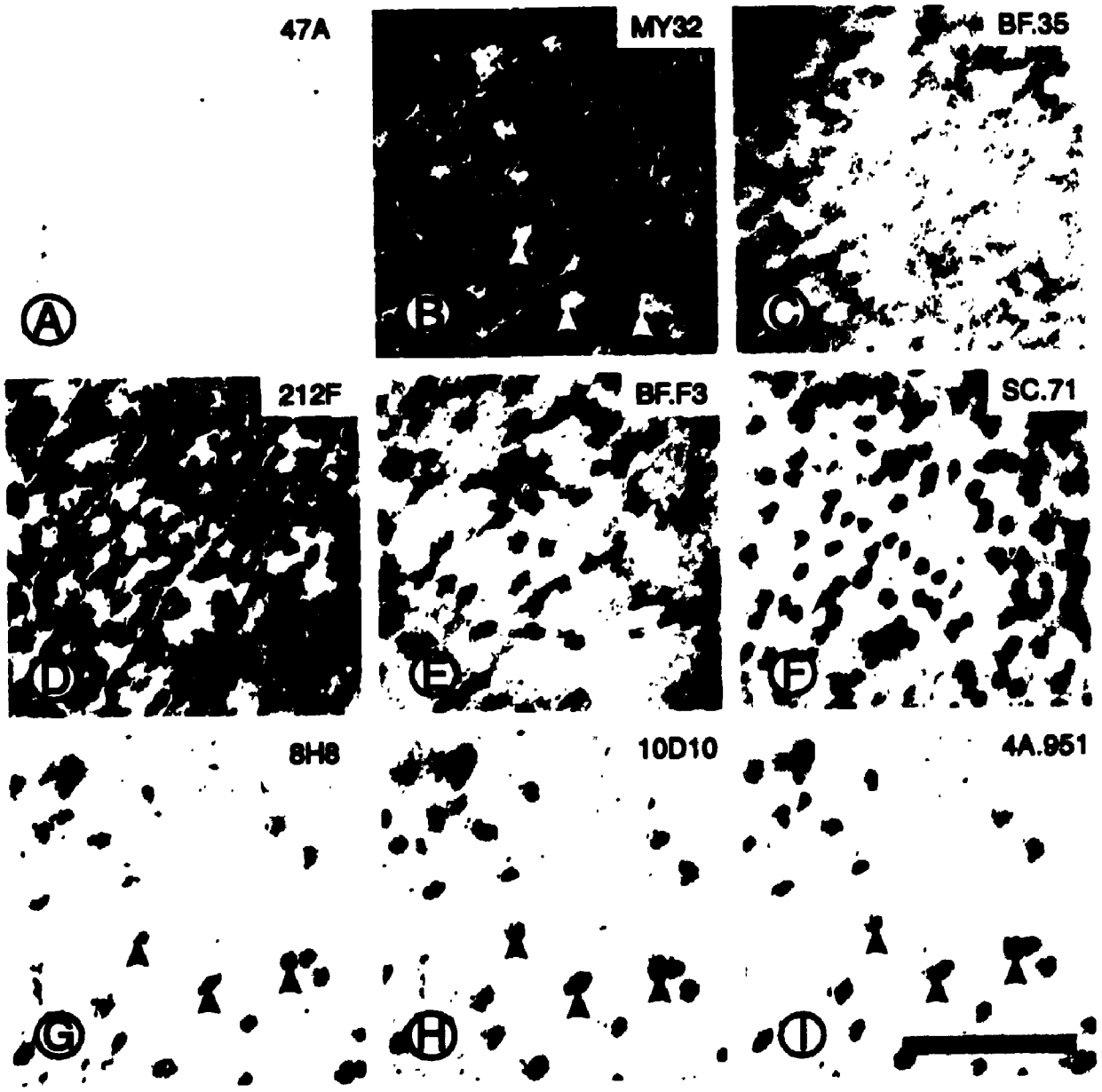
8H8
10D10
4A.951
G
H
I

Figure 6.4 Pattern of MyHC expression in the regenerating tibialis anterior 28 days after injection with marcain. MyHC expression was examined using ABC-AP immunolocalization with MyHC - specific Mabs against embryonic (A;47A), neonatal/adult fast (B;MY-32), all isoforms except IIX and embryonic MyHCs (C;BF.35), fast IIB/IIX (D;212F), fast IIB (E;BF.F3), fast IIA (F;SC.71), all slow (G;8H8) and neonatal/adult slow (H;10D10), and adult slow (I;4A.951) MyHCs. Embryonic MyHC (I) is no longer expressed in the area that had undergone regeneration (as evidenced by the presence of India Ink between the fibres). Neonatal and adult fast isoforms are detected by staining for MY-32 (A), and all of these fibres stain for either fast IIB/IIX (B) or fast IIA (C and D), suggesting that they are mature. All slow staining fibres (➤) show reactivity to all slow antibodies (E-H) indicating that they have fully matured. This suggests that normal adult muscle fibre type is established in the tibialis anterior by 28 days after injection with marcain. Bar = 450 μm.


47A
MY32
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212F
BF.F3
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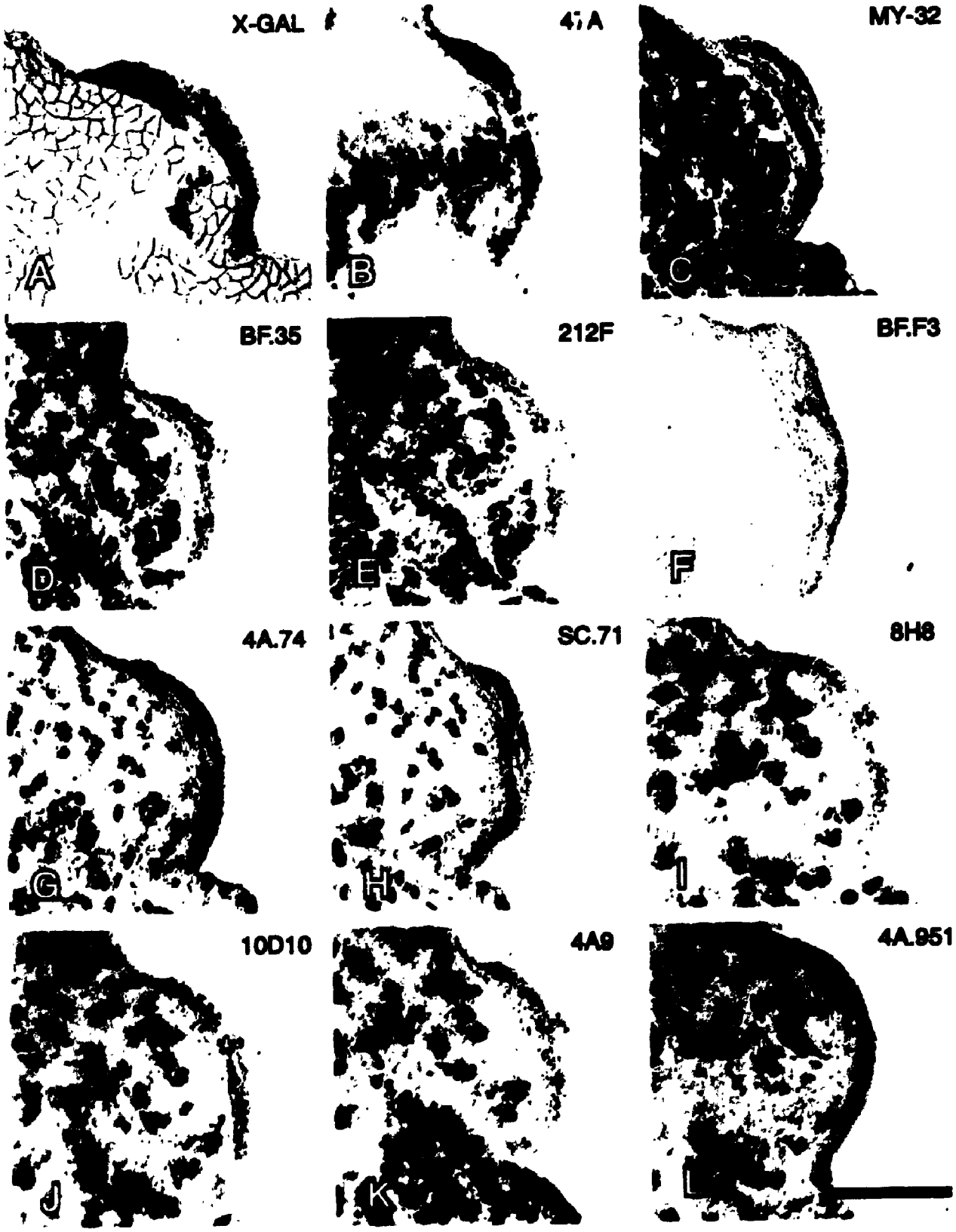
6.3.2 Characterization of L6BAG-A4 derived homotypic fibres after injection into regenerating adult muscle of Wistar Furth rats

To study the effects of varying environments on the maintenance of the L6 phenotype, several different muscles were targeted for injection. The white gastrocnemius is a typically fast muscle (Armstrong and Phelps, 1984) composed predominantly of IIB fibres. The tibialis anterior and plantaris muscles represent typically mixed muscles containing all adult fibre types, while the red region of the gastrocnemius and the soleus muscle represent typically slow muscles (Armstrong and Phelps, 1984). These last two muscles contain large numbers of slow and IIA fibres and small numbers of IIX fibres, and therefore represent muscle environments in which maintenance of the L6 phenotype may be most challenged. Following injection, L6 myoblasts form both homotypic and heterotypic fibres. Homotypic fibres result from the fusion of donor cells with each other to form new myotubes which show intense staining with X-gal, indicating high levels of ß-gal expression, and centrally located nuclei characteristic of regenerating fibres (Benoit and Beli, 1970). These myotubes are usually located outside muscle fascicles, often grouped in small clusters at the periphery of the muscle bed. Heterotypic fibres, which result from the fusion of donor myoblasts with host myoblasts or muscle fibres, show considerably weaker X-gal staining, with peripheral nuclei and a typical polygonal shape on cross section. Heterotypic fibres are discussed in more detail in Chapter 7.

Homotypic fibres were evident in the plantaris muscle seven days after injection with the L6BAG-A4 myoblasts (Fig. 6.5), where peripheral clusters of myotubes outside of the muscle bed were observed (*) usually embedded in either the perimysium or epimysium surrounding muscle fascicles. In some cases large accumulations of undifferentiated cells were observed at the site of injection, however, these were not due to the uncontrolled proliferation of L6BA-A4 cells, since no labelling occurred upon characterization using X-gal immunohistochemistry. These myotubes were darkly stained with X-gal and showed a typically small, cir-

Figure 6.5 Pattern of MyHC expression in L6BAG-A4 homotypic fibres seven days after injection of L6BAG-A4 myoblasts into the plantaris muscle. Serial sections were either stained with X-gal (A) or analyzed for MyHC expression using immunohistochemistry with Mabs specific for embryonic (B;47A), neonatal/adult fast (C;MY-32), all isoforms except IIX and embryonic (D;BF.35), fast IIB/IIX (E;212F), fast IIB (F;BF.F3), fast IIA (G;4A.74 and H;SC.71), all slow (I; 8H8), neonatal/adult slow (I;10D10 and J;4A9), and adult slow (H;4A.951) MyHCs. Staining with X-gal (A) reveals the presence of darkly staining, predominantly donor - derived myotubes at the periphery of the muscle (*). These myotubes react strongly with 47A (for embryonic MyHC) (B), and only very slightly with MY-32 (C). Although 212F (E) does not stain these myotubes, neither does any other Mab including BF.35 (D), suggesting that the second isoform recognized by MY-32 may be adult fast IIX MyHC. The dark spots in the injection site are from the India ink injected with the L6BAG-A4 myoblasts. Bar = 370 μm.


X-GAL
4;A
MY-32
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B
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212F
BF.F3
D
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F
4A.74
SC.71
8H8
G
H
I
10D10
4A9
4A.951
J
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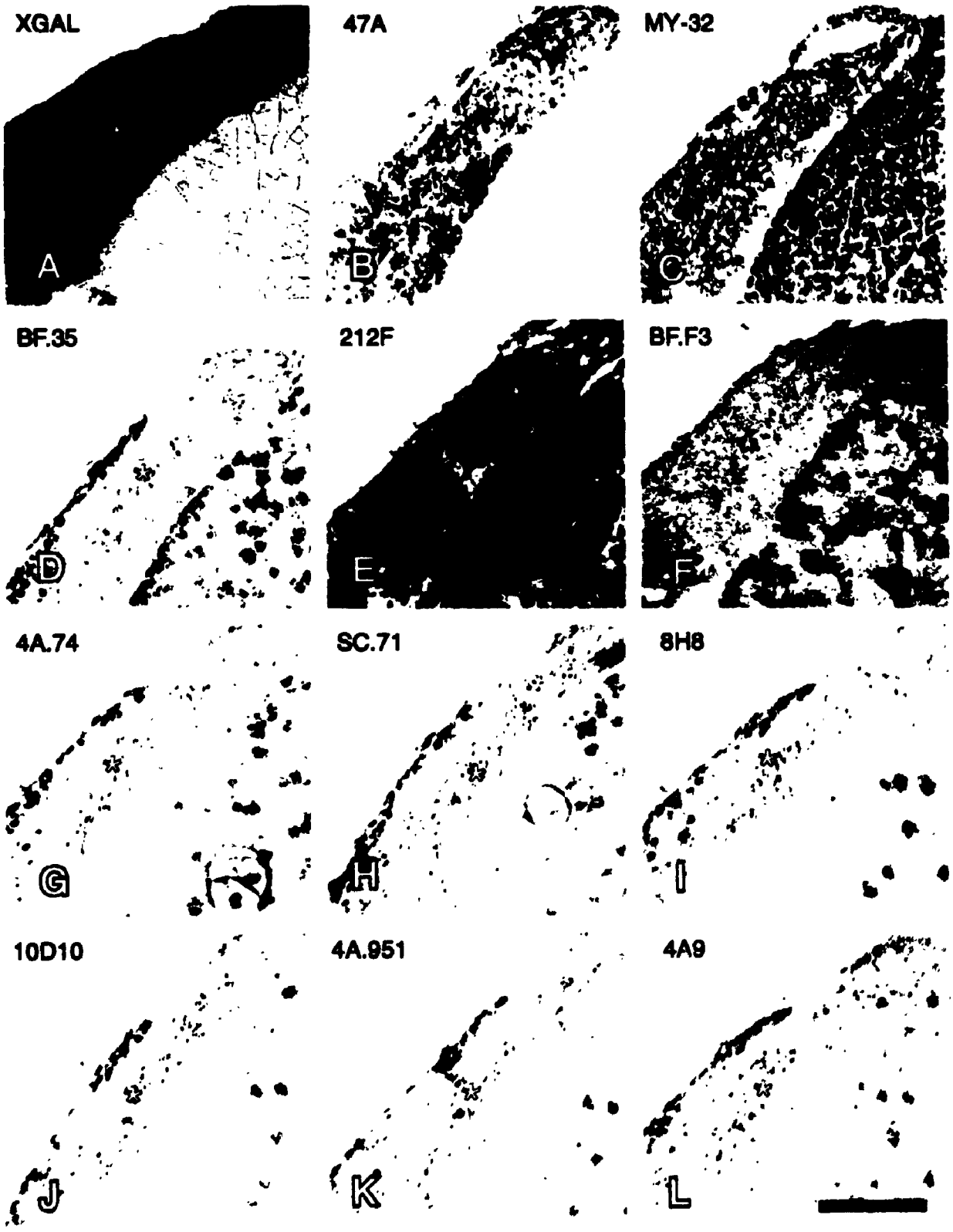
L

cular shape in cross section. These myotubes extended up to several centimeters, but they did not appear to extend along the entire muscle. Upon staining with nuclear dyes, central nucleation was observed (not shown here). Sections next to those that contained concentrations of homotypic fibres were subsequently analyzed for their MyHC expression using ABC-AP immunolocalization . Based on the fibre type profile in the area adjacent to the injection site, it appeared that cells, in many cases, were delivered into a typically mixed muscle area. All myotubes examined at seven days after injection showed a positive reaction with 47A, typical of both L6 myotubes *in vitro* (see Chapter 5), and muscle fibres undergoing regeneration (discussed earlier). These fibres showed only a limited reaction to MY-32, and no reaction with BF.35, indicating that neonatal MyHC is not present. This result is markedly different than the MyHC profile observed during muscle regeneration in the sham-injected contralateral limbs. Since no other fast or slow - specific antibodies detected any other MyHC isoforms, it seemed likely that MY-32 was recognizing very small quantities of IIX MyHC expression that were below the level of detection of 212F.

Examination of homotypic fibres in the plantaris 28 days after injection with X-gal histochemistry (Figure 6.6) indicated that the shape, size and staining intensity characteristic of homotypic fibres was maintained. These fibres still remained localized towards the periphery of the muscle bed outside the normal muscle fascicle (*). Once again, it appeared that the myoblasts were delivered into a typically mixed area of the plantaris muscle. ABC-AP immunolocalization using MyHC specific Mabs revealed that, in addition to expression of embryonic MyHC (evidenced by 47A staining), there was also a second isoform that was recognized by both MY-32 and 212F in the majority of the homotypic myotubes. Negative reactions for both BF.35 and BF.F3 indicated that these Mabs were recognizing a fast IIX MyHC, typical of L6 expression *in vitro*. Fast IIA MyHC was not expressed in these myotubes since neither SC.71 or 4A.74 showed a positive reaction. Homotypic L6BAG-A4 myotubes also failed to react to any of the slow Mabs. Thus, the pattern

Figuure 6.6 Pattern of MyHC expression in L6BAG-A4 homotypic fibres 28 days after injection of L6BAG-A4 myoblasts into the plantaris muscle. Serial sections were either stained with X-gal (A) or analyzed for MyHC expression using immunohistochemistry with Mabs specific for embryonic (B;47A), neonatal/adult fast (C;MY-32), all isoforms except IIX and embryonic (D;BF.35), fast IIB/IIX (E;212F), fast IIB (F;BF.F3), fast IIA (G;SC.71 and H;4A.74), all slow (I; 8H8), neonatal/adult slow (I;10D10 and J;4A9), and adult slow (H;4A.951) MyHCs. Staining with X-gal (A) reveals the presence of darkly stained, predominantly donor - derived myotubes at the periphery of the muscle (*). Most of these fibres still stain lightly for 47A (B) indicating the presence of embryonic MyHC. These fibres also stain very strongly for 212F (E) and MY-32 (C) but not for BF.35 (D), indicating that fast IIX MyHC is also expressed in these myotubes. No other Mabs show reactivity suggesting that IIA, IIB and slow type MyHCs are not expressed in these myotubes. The dark spots in the injection site are from the India ink injected with the L6BAG-A4 myoblasts. Bar = 270 μm.


XGAL
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47A
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MY-32
C
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212F
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BF.F3
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4A.74
G
SC.71
H
8H8
I
10D10
J
4A.951
K
4A9
L

of MyHC expression in homotypic L6BAG-A4 myotubes *in vivo* was remarkably similar to their MyHC profile *in vitro*, with embryonic and adult fast IIX MyHCs being the only isoforms expressed. However, starting at 28 days after injection, homotypic myotubes could also be detected in which embryonic MyHC was no longer expressed, unlike the *in vitro* pattern of expression.

Further examination with X-gal histochemistry in the plantaris muscle 56 days after injection revealed that the size, staining intensity, and peripheral location of homotypic L6BAG-A4 muscle fibres were maintained for the duration of the experiment (Figure 6.7). ABC-AP immunolocalization with the various MyHC specific Mabs demonstrated that the targeted area of the plantaris was made up of almost exclusively IIB fibres. However, examination of the MyHC profile of the L6 myoblast derived homotypic fibres still revealed positive reactions for 47A, MY-32 and 212F. Since the myotubes did not stain for BF.F3, Mab 212F must be recognizing IIX MyHC. However, there was also a positive reaction for BF.35, which was not observed previously, which suggested that BF.35 was either recognizing a third MyHC isoform or was cross reacting to fast IIX or embryonic MyHC. Since IIA or slow MyHC - specific Mabs did not recognize donor myotubes, these cells most likely expressed low levels of neonatal fast MyHC. All myotubes which reacted with 212F also reacted with MY-32, but there were examples where 47A staining was absent indicating a transition of MyHC expression from embryonic to fast IIX MyHC in a subset of homotypic fibres.

Similar characterizations of homotypic L6BAG-A4 myotubes demonstrated similar patterns of expression in all homotypic fibres, regardless of the muscle injected. While embryonic MyHC was the predominant isoform early in differentiation, fast IIX MyHC was upregulated over time, and gradually replaced the developmental isoform. These observations are summarized in Tables 6.1 and 6.2. One example of this was observed in the tibialis anterior 28 days after injection using ABC-AP immunolocalizations (Figure 6.8). X-gal staining revealed an injection site (*) that lay in the perimysium between muscle fascicles. These myo-

Figure 6.7 Pattern of MyHC expression in L6BAG-A4 homotypic fibres 56 days after injection of L6BAG-A4 myoblasts into the plantaris muscle. Serial sections were either stained with X-gal (A) or analyzed for MyHC expression using immunohistochemistry with Mabs specific for embryonic (B;47A), neonatal/adult fast (C;MY-32), all isoforms except IIX and embryonic (D;BF.35), fast IIB/IIX (E;212F), fast IIB (F;BF.F3), fast IIA (G;SC.71 and H;4A.74), all slow (I; 8H8), and adult slow (H;4A.951) MyHCs. Staining with X-gal (A) reveals the presence of darkly staining, predominantly donor - derived myotubes at the periphery of the muscle (*). The majority of these fibres still stain for 47A (B) indicating the persistence of the embryonic MyHC. Both 212F (E) and MY32 (C) stain these fibres as well, while BF.F3 (F) and SC.71(G) show no reactivity, indicating the presence of fast IIX MyHC. Some of these fibres (→) show a positive reaction to the fast antibodies only indicating that in some cases the myotubes stop expressing embryonic MyHC. A weak reaction is also seen for 4A.74 (H), which suggests that the level of IIX MyHC is high enough for the Mab to cross react (as seen previously). BF.35 (D) appears to recognize some homotypic fibres at the periphery of the injection site. Due to the lack of reactivity of the other fast and slow specific isoforms, it seems likely that this may represent the appearance of fast neonatal MyHC in these fibres. The dark spots in the injection sites are from the India ink injected with the L6BAG-A4 myoblasts. Bar = 130 μm.

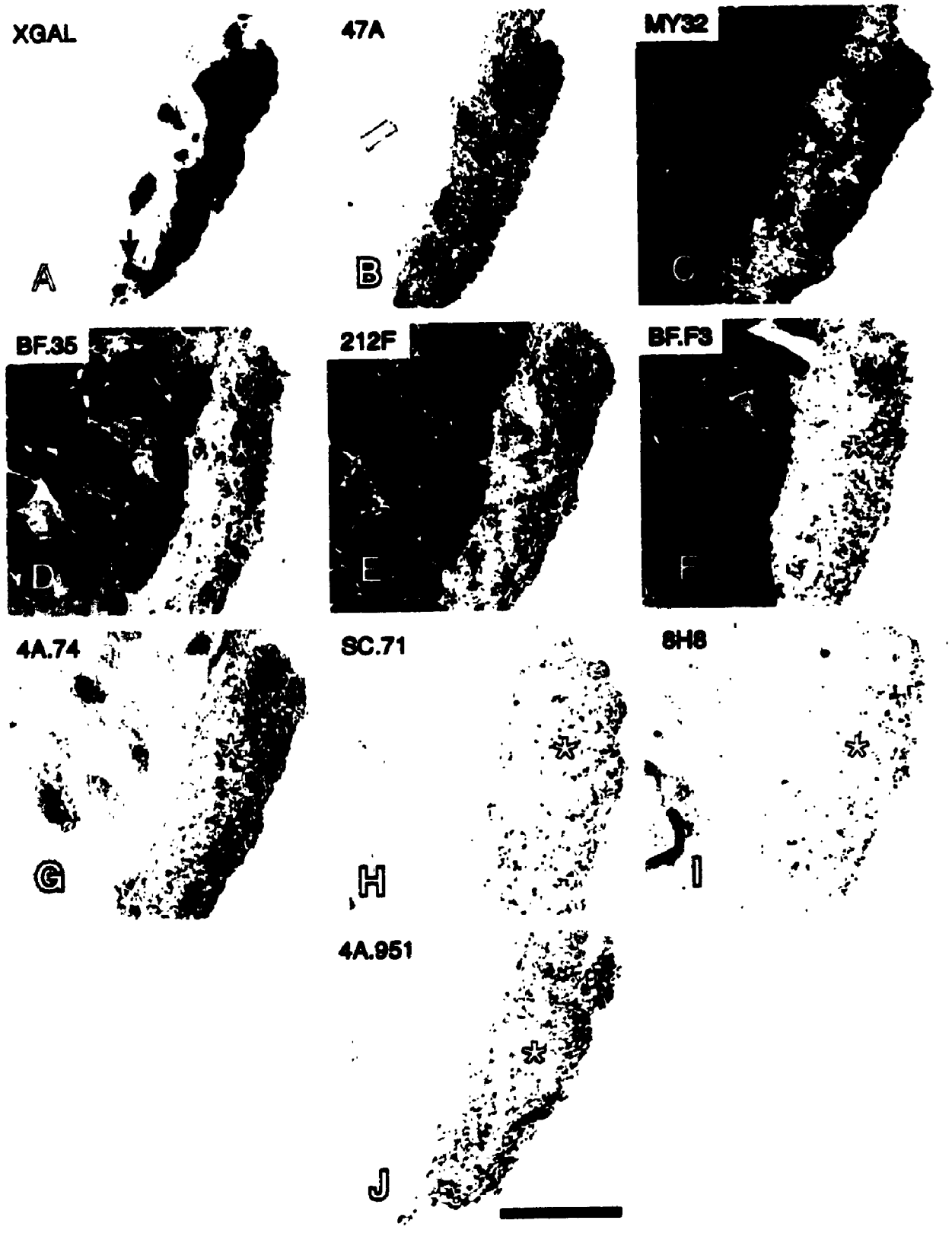

XGAL
A
47A
B
MY32
C
BF.35
D
212F
E
BF.F3
F
4A.74
G
SC.71
H
8H8
I
4A.951
J

Figure 6.8 Pattern of MyHC expression in L6BAG-A4 homotypic fibres 28 days after injection of L6BAG-A4 myoblasts into the tibialis anterior muscle. Serial sections were either stained with X-gal (A) or analyzed for MyHC expression using immunohistochemistry with Mabs specific for embryonic (B;47A), neonatal/adult fast (C;MY-32), all isoforms except IIX and embryonic (D;BF.35), fast IIB/IIX (E;212F), fast IIB (F;BF.F3), fast IIA (G;SC.71), neonatal/adult slow (H;10D10) and adult slow (I;4A 951) MyHCs. Staining with X-gal (A) reveals the presence of darkly staining, predominantly donor - derived myotubes at the periphery of the muscle (*). Most of these fibres still stain lightly for 47A (B) indicating the presence of embryonic MyHC. These fibres also stain very strongly for MY-32 (C), but only lightly for BF.35(D) and 212F (E). These homotypic fibres do not react with BF.F3 (F) or SC.71 (G) indicating that fast IIX MyHC and possibly neonatal fast MyHCs are expressed in these myotubes. Mabs 10D10 (H) and 4A.951 (I) against slow MyHC do not react with these myotubes. The dark spots in the injection site are from the India ink injected with the L6BAG-A4 myoblasts. Bar = 260 μm.

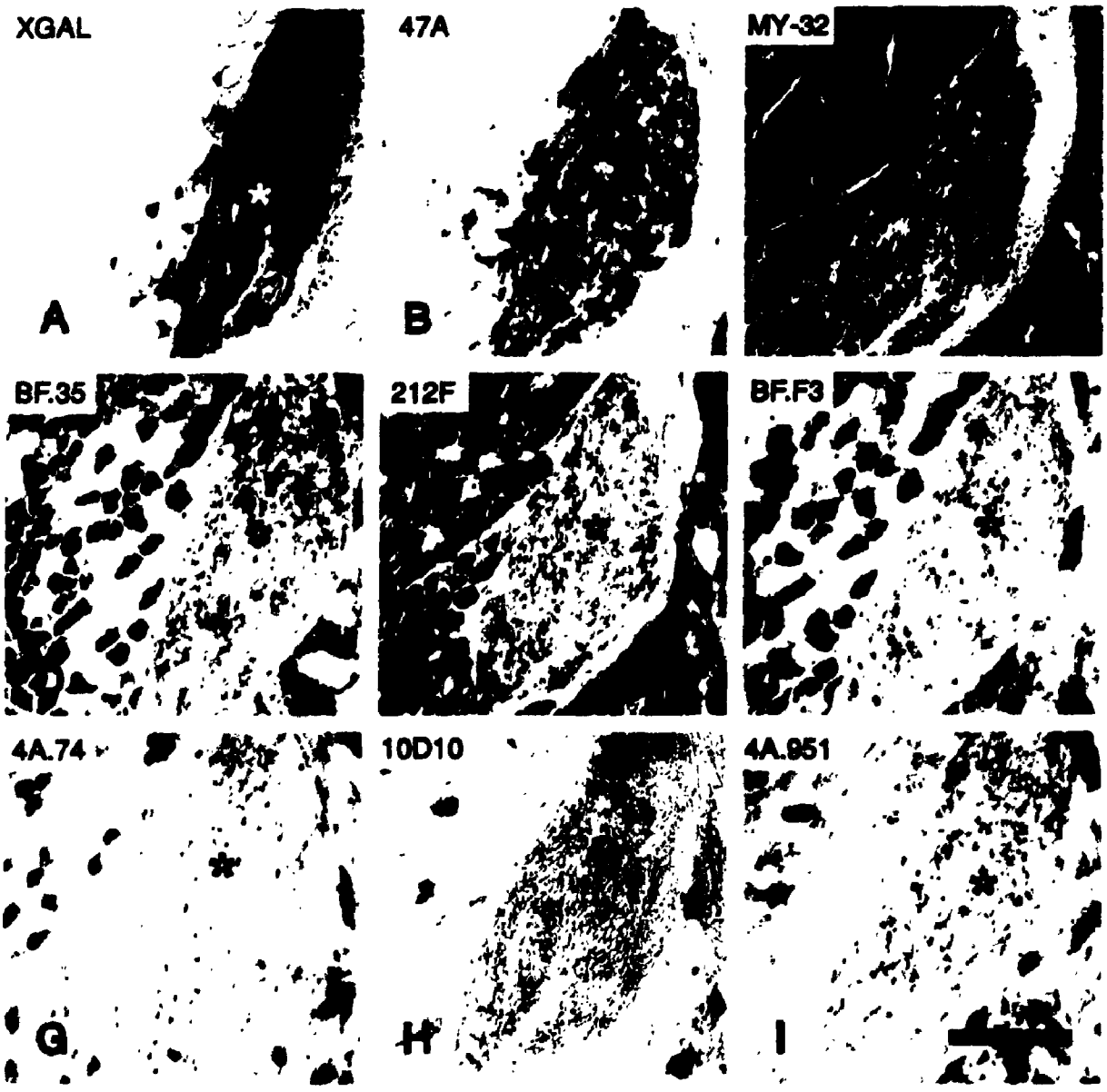

XGAL
47A
MY-32
A
B
BF.35
212F
BF.F3
4A.74
10D10
4A.951
G
H
I

Table 6.1 Reactivity of monoclonal antibodies to homotypic L6 myotubes *in vivo*

Muscle	Weeks Post - Injection	Monclonal Antibodies Tested										
		47A	MY-32	BF.F3	212F	SC.71	4A.74	8H8	10D10	4A9	4A.951	BF.35
Tibialis Anterior	One	+	-	-	-	-	-	-	-	-	-	-
	Two	+	+	-	+	n.d.	-	-	-	-	-	-
	Four	+	+	-	+	-	--	-	-	-	-	+
	Eight	+	+	-	+	-	-	+	-	-	-	+
Gastroc.	One	+	-	-	-	n.d.	-	-	-	-	-	-
	Two	+	-	-	-	n.d.	n.d.	-	-	-	-	-
	Four	+	+	-	+	-	+	-	-	-	-	-
	Eight	+	+	-	+	-	+	-	-	-	-	n.d.
Soleus	One	+	-	-	-	-	-	-	-	-	-	-
	Two	+	+	-	+	-	-	-	-	-	-	-
	Four	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Eight	+	+	n.d.	+	n.d.	-	-	-	-	n.d.	n.d.
Plantaris	One	+	-	-	-	-	-	-	-	-	-	-
	Two	+	+	-	+	-	-	-	-	-	-	-
	Four	+	+	-	+	-	-	-	-	-	-	-
	Eight	+	+	-	+	-	-	-	-	-	-	+

n.d. - not determined

Table 6.2 Myosin heavy chain expression of homotypic L6BAG-A4 myotubes

Weeks After Injection	Muscle	Myosin Heavy Chain Expression					
		Embryonic	Neonatal	IIA	IIB	IIX	Slow
1	Gastroc.	+++	-	-	-	-	-
	Soleus	+++	-	-	-	+	-
	Plantaris	+++	-	-	-	-	-
	TA	+++	-	-	-	-	-
2	Gastroc.	+++	-	-	-	+	-
	Soleus	+++	-	-	-	+	-
	Plantaris	+++	-	-	-	+	-
	TA	+++	-	-	-	+	-
4	Gastroc.	++	-	-	-	++	-
	Soleus	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Plantaris	+++	-	-	-	+++	-
	TA	++	+	-	-	+++	-
8	Gastroc.	++	-	-	-	+++	-
	Soleus	++	n.d.	-	n.d.	++	-
	Plantaris	++	+	-	-	+++	-
	TA	++	+	-	-	+++	-

+++ - ≥ 90% of myotubes are positive; ++ - ≤ 90% of myotubes are positive; + - ≤ 5% of myotubes are positive

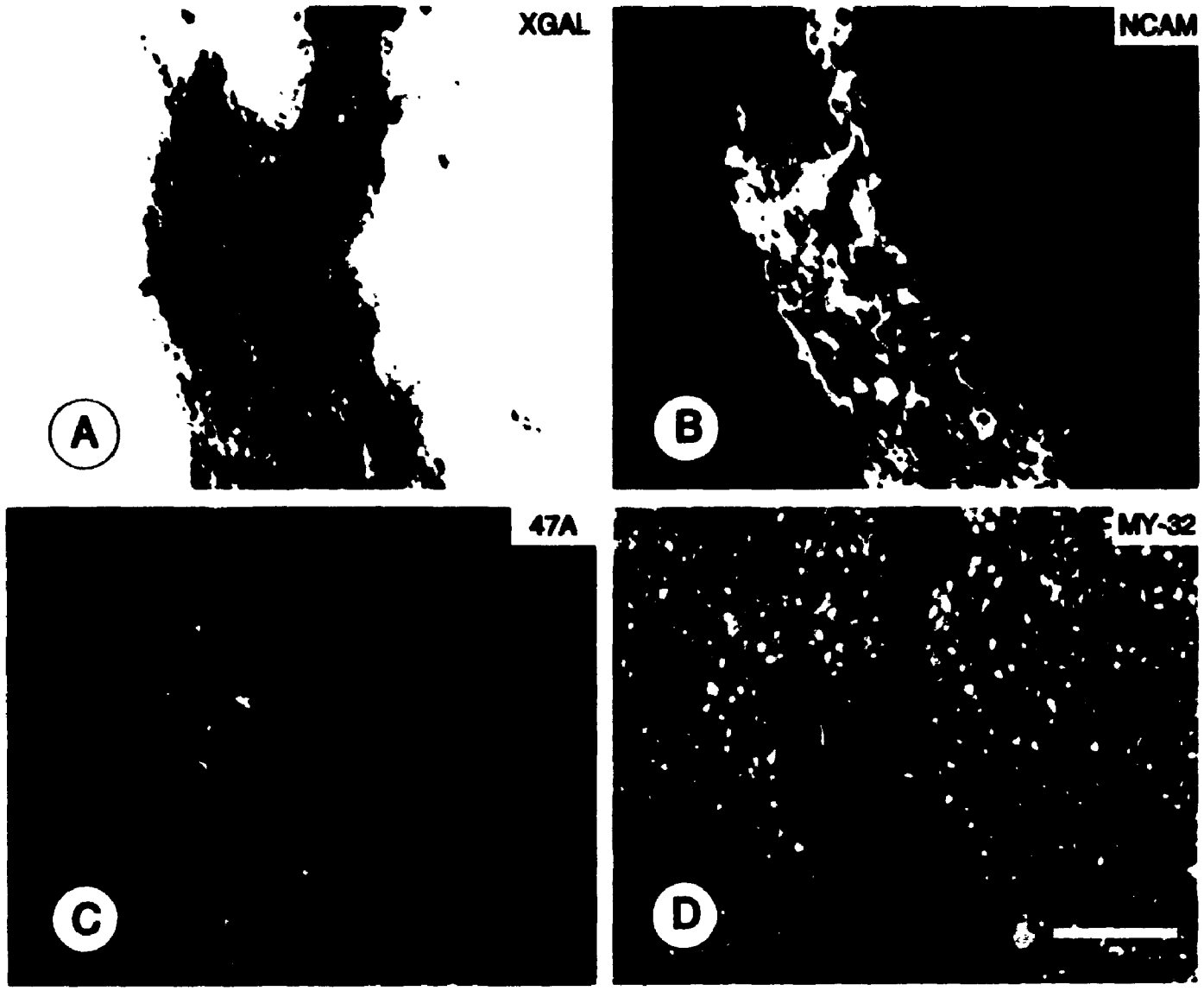
tubes exhibited the same pattern of MyHC expression observed in homotypic L6BAG-A4 myotubes in the plantaris. 47A, MY-32 and 212F all showed a positive reaction to the myotubes. Once again several MY-32 positive myotubes were not recognized by Mab 47A, indicating that they had undergone some kind of transition, down-regulating embryonic MyHC in favour of fast IIX MyHC. All of the other Mabs showed no reaction to the homotypic myotubes, with the exception of BF.35, which showed a light reaction. This supported the idea that a third MyHC isoform was being expressed in these myotubes, possibly the neonatal fast MyHC.

6.3.3 Characterization of NCAM expression in homotypic L6BAG-A4 myotubes and its relationship to MyHC expression

MyHC characterizations described in the previous section documented a transition in the phenotype of L6BAG-A4 myotubes *in vivo*. Two changes occurred in the MyHC profile of these myotubes with embryonic MyHC expression being down-regulated, and fast IIX MyHC expression was up-regulated. To examine the role of innervation in this transition, a polyclonal rabbit antibody, specific for all NCAM isoforms, was colocalized with the various MyHC antibodies shown previously to react positively to the myotubes *in vivo*. The expression of NCAM is known to be expressed along the entire surface of myotubes prior to innervation. Upon innervation, NCAM becomes localized to the motor endplate region only. Therefore, myotubes that are NCAM negative are presumably innervated while those that show punctate staining along the membrane are not innervated (see section 1.2.5).

Injection of L6BAG-A4 myoblasts into the regenerating extensor digitorum longus of adult Wistar Furth rats produced an area of myotubes within the perimysium between adjacent muscle fascicles (Figure 6.9). X-gal immunohistochemistry revealed these myotubes expressed high levels of β-gal and had a circular cross sectional shape, typical of donor derived myotubes. Upon fluorescent co-labelling of serial sections using an NCAM specific polyclonal anti-

Figure 6.9 NCAM expression of homotypic L6BAG-A4 myotubes seven days after injection into the extensor digitorum longus muscle. Serial sections were either stained for X-gal (A) or analysed using immunofluorescence with an NCAM specific polyclonal antibody (B), or Mabs specific for embryonic (C;47A) or neonatal/adult fast (D;MY-32) MyHCs. Primary antibodies were identified by fluorescein (NCAM and 47A) or rhodamine - conjugated secondary antibodies (MY-32). At one week after injection the X-gal homotypic myotubes are positive for both NCAM (B) and 47A (C), but not for MY-32 (D). This indicates that these myotubes are early in their differentiation and are not innervated. Bar = 110 μm.


XGAL
A
NCAM
B
47A
C
MY-32
D

body in conjunction with 47A, embryonic MyHC and NCAM were essentially coextensive. This suggested that donor derived myotubes were not innervated at this early time point after injection. Coreactivity of the two antibodies to muscle fibres outside the injection site indicated that the host fibres were undergoing regeneration, a process that involves both denervation and re-expression of developmental isoforms. Fluorescent immunolocalization with MY-32 revealed that homotypic myotubes at this time after injection did not express neonatal or adult fast MyHCs, as expected. The only colabelling of NCAM and MY-32 occurred in host muscle fibres. This was not surprising since these fibres undergo normal regeneration, in which neonatal MyHC is usually expressed.

Examination of these myotubes under higher magnification supported the observation that homotypic L6BAG-A4 myotubes were NCAM positive and expressed only embryonic MyHC (Figure 6.10). Although several myotubes (➤) stained for both NCAM and MY-32, these fibres were not X-gal positive, suggesting that they were host-derived, regenerating muscle fibres. Similar examination revealed that all of the putative homotypic, donor-derived myotubes which stained strongly with X-gal, expressed both NCAM and embryonic MyHC. The fact that donor derived myotubes were not innervated at this time point after injection was not surprising. The finding that only embryonic MyHC was expressed in these myotubes, supported the results described in the preceding section.

To determine if the disappearance of 47A reactivity to L6BAG-A4 myotubes *in vivo* is related to these fibres becoming innervated, injection sites in the red gastrocnemius and tibialis anterior were analyzed for NCAM and embryonic MyHC expression at 28 (Figure 6.11) and 56 days (Figure 6.12) after injection. X-gal histochemistry of the red gastrocnemius muscle 28 days after injection revealed several typical donor - derived myotubes in extensive connective tissue at the periphery of the muscle. Examination of these specific myotubes using fluorescent colocalization of 47A and NCAM at low and high magnification, revealed two homotypic myotubes that were both positive for embryonic MyHC. However, the

Figure 6.10 Immunofluorescent localization of NCAM and MyHC expression in homotypic L6BAG-A4 fibres seven days after injection into the extensor digitorum longus muscle photographed at high magnification. Serial sections were either stained for X-gal (A,B) or analysed using immunofluorescence with an NCAM specific polyclonal antibody (C,D), or Mabs specific for neonatal/adult fast (E;MY-32) or embryonic (F;47A) MyHCs. Primary antibodies were identified by fluorescein (NCAM and 47A) or rhodamine - conjugated secondary antibodies. Individual homotypic myotubes (→), determined by their X-gal expression (A,B), are all NCAM (C,D) positive, indicating that they are not innervated. These fibres express embryonic MyHC (F) but not fast IIX (E) MyHC at this time. Bar = 65 µm.

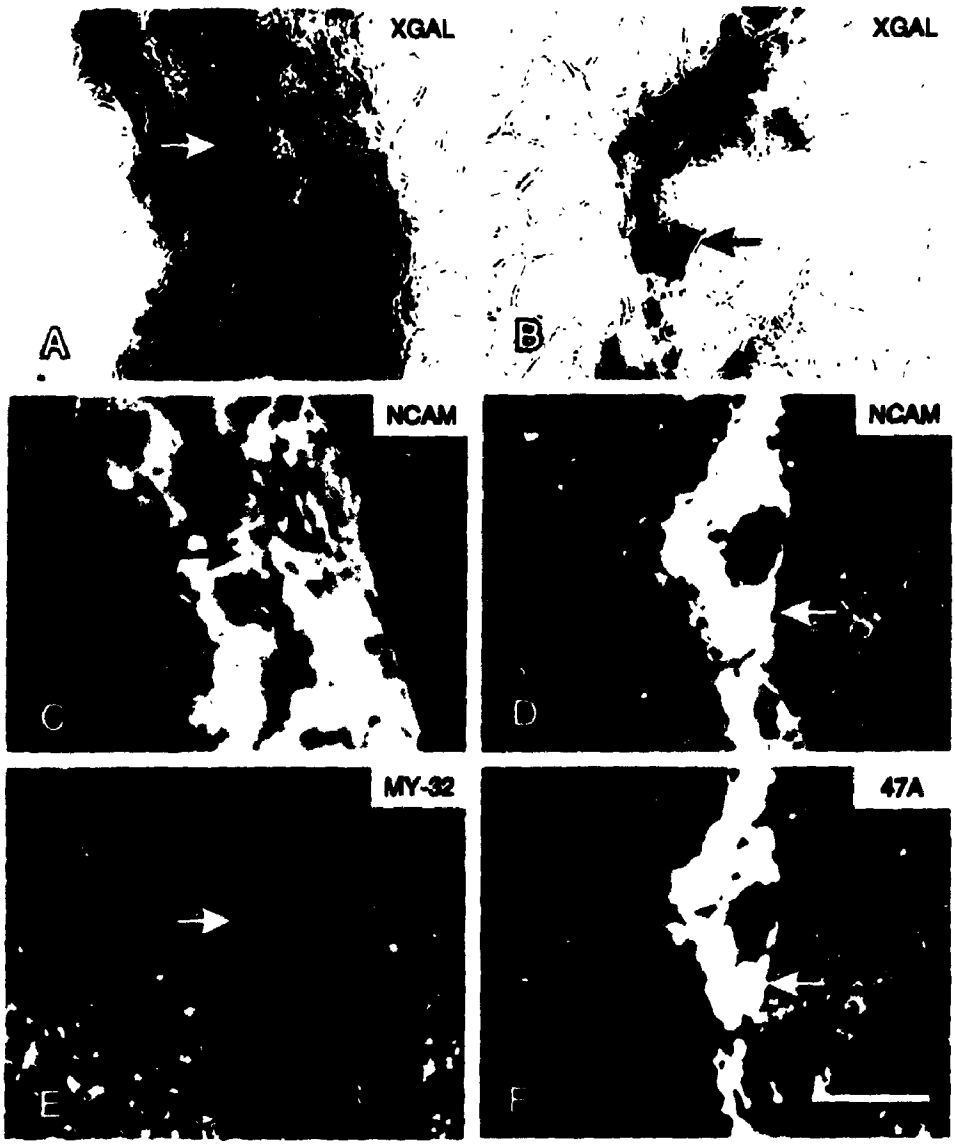

XGAL
XGAL
A
B
NCAM
NCAM
C
D
MY-32
47A
E
F

Figure 6.11 NCAM expression of homotypic L6BAG-A4 myotubes 28 days after injection of L6BAG-A4 myoblasts into the medial gastrocnemius muscle of adult Wistar Furth rats. Immunofluorescent co-localization with antibodies against NCAM (A,C) and embryonic MyHC (47A;B,D)is shown at both low (A,B) and high (C,D) magnifications. Primary antibodies were recognized by secondary antibodies conjugated to fluorescein (NCAM) or rhodamine (47A). Adjacent sections were stained for X-gal and photographed at high magnification under phase contrast (E) or bright field (F) optics, to identify predominantly homotypic myotubes. Two such myotubes (➤) both stain for 47A (B,D) indicating embryonic MyHC expression. The lower fibre appears to be innervated since it does not express NCAM staining (A,C). The presence of embryonic MyHC within innervated myotubes suggests that innervation is not involved in its downregulation. Bar = 100 μm for A and B, 45 μm for C, D, E and F.

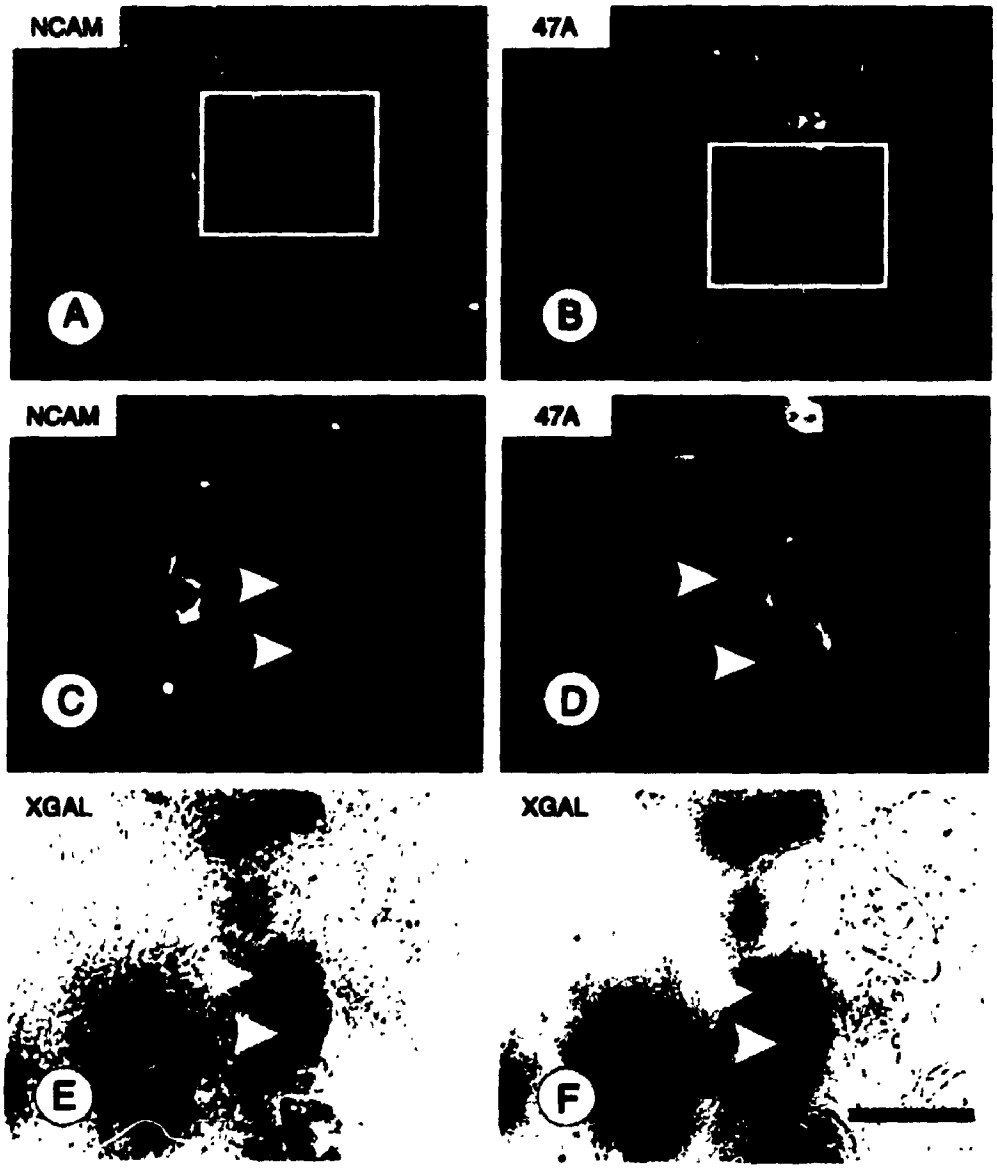
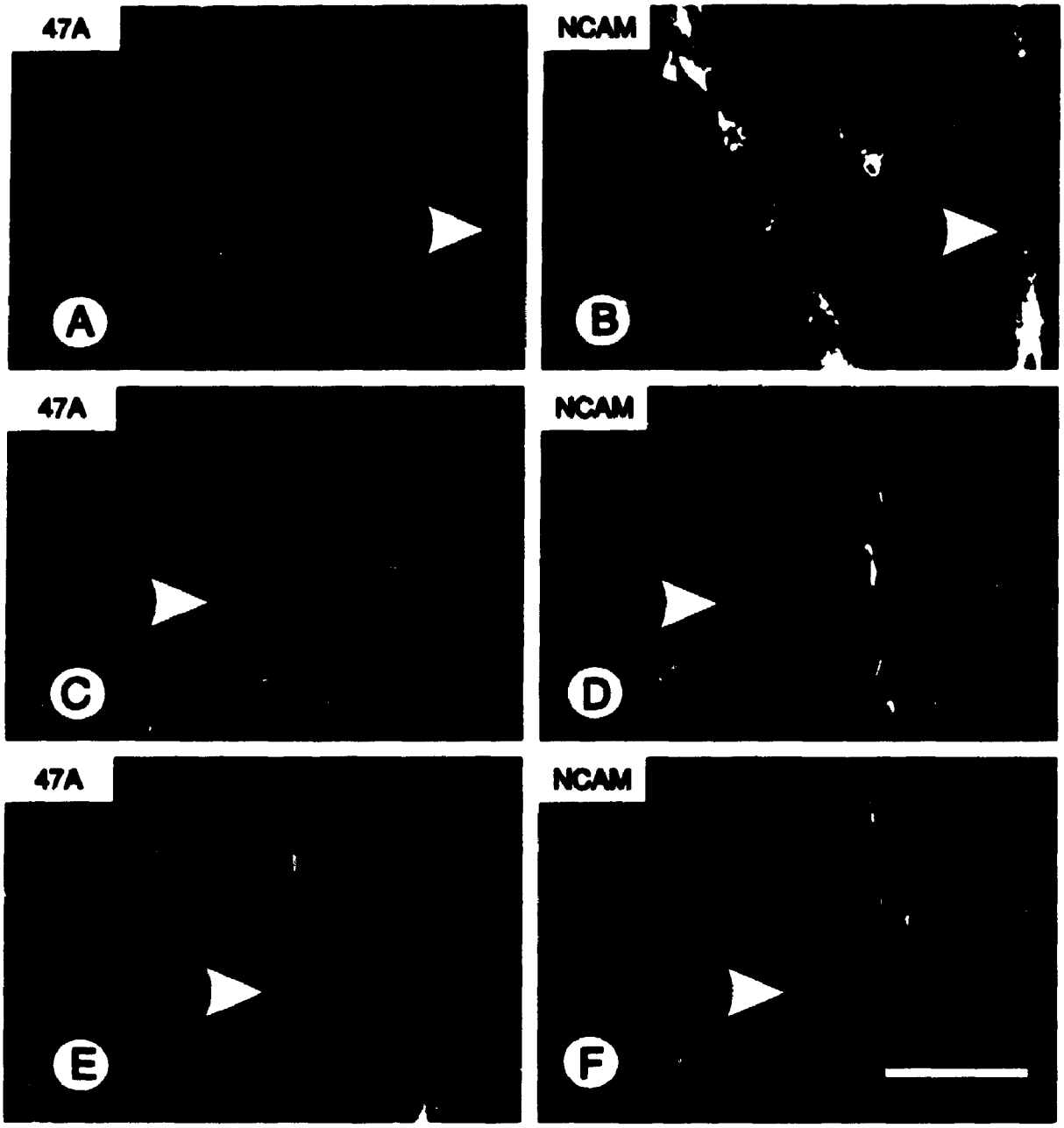

NCAM
47A
A
B
NCAM
47A
C
D
XGAL
XGAL
E
F

Figure 6.12 NCAM expression of homotypic L6BAG-A4 myotubes 56 days after injection of L6 myoblasts into the tibialis anterior muscle of adult Wistar Furth rats. Immunofluorescent co-localizations were carried out with antibodies against embryonic MyHC (47A;A,C,E) and NCAM (B,D,F). Primary antibodies were recognized by secondary antibodies conjugated to fluorescein (NCAM) or rhodamine (47A). Myotubes that express NCAM but not embryonic MyHC can be seen in A and B, while myotubes that stain for embryonic MyHC but not NCAM can be seen in C and D, and E and F. All myotubes of interest are labelled (➤). The lack of coordination between the expression of embryonic MyHC and NCAM strongly suggests that innervation is not involved in the downregulation of embryonic MyHC. Bar = 75 μm.

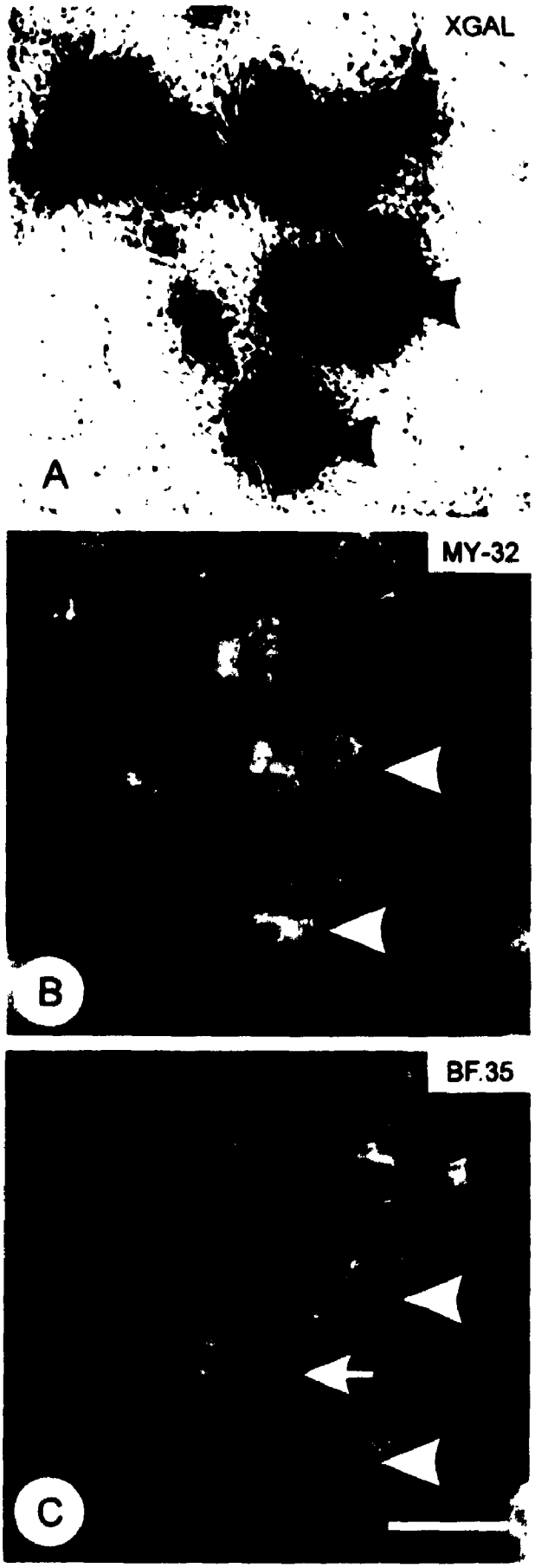

47A
NCAM
A
B
47A
NCAM
C
D
47A
NCAM
E
F

lower myotube did not stain for NCAM indicating that it had become innervated. Although this suggested that innervation did not play a role in embryonic MyHC expression, it was possible that there had been an effect at the mRNA level, which was not detected at the protein level.

Similar localizations at 56 days after injection into the tibialis anterior supported the observation that innervation did not regulate embryonic MyHC expression (Figure 6.12). Fluorescent localizations of 47A and the NCAM specific polyclonal antibody revealed myotubes that were positive for NCAM but not 47A, or negative for NCAM and positive for 47A. Therefore, at 56 days after myoblast injection, there was still a persistence of the embryonic MyHC isoform, even after innervation occurred. The presence of myotubes that no longer stained for 47A but still stained strongly for NCAM could indicate that the down-regulation of embryonic MyHC can precede innervation. Since there was no correlation between NCAM and embryonic MyHC expression, one can conclude that the developmental switch in embryonic MyHC expression was nerve - independent.

To determine if the regulation of IIX MyHC was dependent upon innervation, similar localizations were carried out on homotypic L6BAG-A4 myotubes in the lateral gastrocnemius 28 days after injections (Figure 6.13). X-gal histochemistry was again used to identify putative homotypic myotubes, and serial sections were analyzed using fluorescent double localization of the NCAM specific antibody with either MY-32 (which recognizes all neonatal and adult fast MyHC isoforms), or BF-35 (which recognizes all isoforms except embryonic and adult fast IIX MyHCS). As expected, MY-32, but not BF.35, reacted to the homotypic fibres, indicating the presence of the IIX MyHC isoform. Interestingly, NCAM could be colocalized with all of the myotubes indicating that innervation had still not occurred. Therefore, it appeared that, like embryonic MyHC, the expression of the fast IIX MyHC isoform was not regulated by innervation. These results indicated that the pattern of MyHC expression in L6BAG-A4 homotypic fibres was not governed by innervation, and that the transition in phenotype may be governed by an internal control mechanism.

Figure 6.13 Comparison of MyHC and NCAM expression in homotypic L6BAG-A4 myotubes 28 days after injection into the white gastrocnemius muscle of adult Wistar Furth rats. Serial sections were analyzed for B-galactosidase using X-gal histochemistry (A) or for MyHC / NCAM expression using immunofluorescent co-localization (B,C). NCAM was detected with a rabbit polyclonal antibody using a fluorescein-conjugated secondary antibody. Mabs specific for neonatal/adult fast MyHC (B;MY-32) or all MyHCs except embryonic and IIX (C; BF.35) were detected using a rhodamine conjugated secondary antibody. Localization reveals the myotubes (→) that co-express NCAM with MY-32 (yellow;B), but not BF.35 (green;C). The absence of BF.35 reactivity in these myotubes indicates that they are co-expressing IIX MyHC with NCAM. This suggests that innervation is not associated with the expression of IIX MyHC in L6BAG-A4 homotypic fibres. Bar = 30 μm.


XGAL
A
MY-32
B
BF.35
C

6.4 Discussion

Upon injection of L6BAG-A4 myoblasts into regenerating hindlimb muscles of adult rat, homotypic myotubes, derived predominantly from donor myoblasts, are formed and exhibit a pattern of MyHC expression that is characteristic of the phenotypic expression of L6 myotubes *in vitro*. These myotubes initially express only embryonic MyHC, and this remains the predominant isoform expressed until four weeks post - injection. The myotubes then undergo a transition in which the adult fast IIX MyHC isoform is up-regulated and the embryonic MyHC is gradually down-regulated. Further analysis of these homotypic fibres for NCAM reveals that they become innervated beginning around the same time that this MyHC transition occurs. However, the state of innervation proved to have no relation to the regulation of either embryonic or adult fast IIX MyHC, indicating that these processes may be governed by intrinsic controls.

The introduction of L6BAG-A4 myoblasts into a regenerating muscle environment allows these myoblasts to fuse with host satellite cells and regenerating muscle fibres to form heterotypic muscle fibres. In addition, there is also a large population of cells which remain at the periphery of the muscles or between muscle fascicles to form homotypic myotubes, which are derived predominantly from the injected cells. Since these myotubes maintain the characteristic IIX MyHC expression of L6 cells and down-regulate embryonic MyHC expression, these results support the hypothesis that L6 myoblasts display the unique potential of forming exclusively fast IIX myotubes both *in vitro* and *in vivo*. The observation that myoblasts maintain their characteristic *in vitro* MyHC profile after injection into regenerating muscle supports work recently done in an avian model (DiMario *et al.* 1993; DiMario and Stockdale, 1995), in which primary quail myoblasts of both fast and slow origin were injected into fetal chick muscles. These injections resulted in the formation of homotypic myotubes which maintained their phenotype in all muscle environments examined. However, since these myotubes were only followed ten days *in vivo*, these experiments did not address the possibility of any gradual

effects of the environment. Also, in these chick experiments, the resulting myotubes were not analyzed for their state of innervation. The observation that L6BAG-A4 myotubes do become innervated *in vivo*, and that this innervation does not affect the final phenotype of the myotubes, therefore extends the observations of Stockdale's group. The innervation of these fibres is not surprising considering denervated myotubes have been shown to express increased levels of neurotrophic factors (Oppenheim *et al.* 1993) and higher levels of NCAM (Covault and Sanes, 1985), two factors known to play a role in muscle/nerve interaction and final innervation (Landmesser *et al.* 1988). The observation that these homotypic myotubes become innervated is consistent with experiments that were carried out by Wernig et al (1991), in which putative homotypic fibres reportedly became innervated starting at four weeks after injection of cloned neonatal mouse myoblasts into regenerating mouse muscle. Although this group noticed that a fast fibre phenotype predominated early after injection, they also observed a transition to Type I fibres, suggesting that environmental influences may eventually control the phenotype of the myoblasts. These experiments were limited however, by the fact that the myoblasts used were not characterized *in vitro*, and that the assays used (acid ATPase) were not sensitive enough to delineate between various subtypes of fast fibres.

The examination of myoblast potential using myoblast injection was more rigorously tested by Hughes and Blau (1992), who concluded that the development of fibre types was completely dependent upon the innervation state of the fibre. Unfortunately, the MyHC phenotype of the myoblasts injected, both C2C12 myoblasts and primary satellite cell myoblasts, were not restricted *in vitro*, indicating that an extensive potential may exist for these cells, allowing them to adjust to all muscle environments. The Hughes and Blau experiments also differed from the current study in that donor myoblasts were injected into non-regenerating muscles, and all of the fibres examined were believed to be heterotypic fibres containing nuclei of both donor and host origin. This did not allow for the examination of purely donor myotubes as presented here. A more comprehensive comparison of

Hughes and Blau's work with the L6BAG-A4 heterotypic fibres is in Chapter 7.

The appearance of IIX MyHC as the predominant isoform in these myotubes regardless of the muscle environment, indicates that these cells have a distinct predetermined pattern of expression that can only be partially realized in culture. It could be suggested that these cells are receiving contributions from host satellite cells, which are known to migrate over a large distance to repopulate areas of muscle damage, even from adjacent muscles (Watt *et al.* 1987; Hughes and Blau, 1990). However, if this was the case, one would expect the phenotype exhibited by these cells to be dependent upon the area in which the myotubes develop. Similar injections performed in the present study with L6 cells labelled with a β-galactosidase reporter gene targeted to the nucleus, produced similar putative homotypic myotubes which contained labelled nuclei almost exclusively (data not shown). By extrapolation, the contribution of host satellite cells to homotypic myotubes is probably negligible. One would also expect that the few satellite cells that may fuse to the homotypic fibres would be highly susceptible to external influences, including those from the L6 nuclei, since they have shown variable patterns of MyHC expression based on environmental cues *in vitro* (Düsterhöft and Pette, 1993).

In light of previous experiments in which primary myoblasts injected into the brain developed into mature fibre types in the absence of innervation (Chapter 4), it was not surprizing that innervation was not essential for the up-regulation of the IIX isoform. The observations that the IIX isoform can be expressed in culture (where nerve influences are absent) and during muscle regeneration (where muscle/nerve interactions may have been disrupted) argue against the role of innervation in the regulation of IIX MyHC. Interestingly, it has been shown that adult MyHC isoforms appear following the down-regulation of a polysialylated NCAM (PSA-NCAM) in developing human muscle (Figarella-Branger *et al.* 1992) that is down-regulated prior to innervation. Although the authors do not specifically identify the adult fast MyHC that appears during down-regulation of PSA-NCAM, it is possible that this

isoform is IIX. The IIA isoform can be detected almost immediately after fusion (Cho *et al.* 1994), while the IIB isoform does not normally appear during development until well after innervation has occurred (Whalen *et al.* 1981). Therefore, it may actually be the disappearance of this developmental isoform of NCAM which correlates with the appearance of IIX MyHC, However, a causal relationship between these two events seems unlikely. One possible internal mechanism regulating the expression of IIX MyHC may be changes in the expression of the myogenic regulatory factors (mrf). It has been postulated that differential expression of the mrfs may be involved in the establishment of slow and fast fibre types, with higher amounts of MyoD existing in fast fibres and higher amounts of myogenin in slow fibres (Hughes *et al.* 1993). Although L6 myoblasts do not express MyoD (Hinterberger *et al.* 1991), MRF4 is expressed and it is possible that the IIX MyHC may be linked in some fashion to the MRF4 gene. MRF4 is expressed in L6 myotubes after myf-5, myogenin and embryonic MyHC are expressed, and at the same time that IIX MyHC is first detected. However, to properly address this issue, it would be necessary to examine mature muscle in the absence of MRF4 production (ie. knock-out mice). Although there is evidence that a particular embryonic isoform in slow chick muscles disappears upon innervation (Gao and Kennedy, 1992), most of the literature agrees with the conclusion that the down-regulation of embryonic MyHC is not tied to the innervation state of the muscle (Figarella-Branger *et al.* 1992).

An alternative explanation for the inability of innervation to reprogram L6 myotubes may be that the L6 myotubes are attracting the motoneurons appropriate for IIX fibres. For this to be true, the appropriate nerve terminal must be present in the vicinity of the donor myotubes. This seems unlikely for injection sites are within, or adjacent to typically slow muscles (ie. medial gastrocnemius and soleus) in which no IIX fibres exist, since it is unlikely that motoneurons located there would be appropriate for IIX fibres. Therefore, there either must be some sort of trophic signal expressed by the myotube to specifically attract the appropriate motoneuron

from outside the area, or the motoneurons within the area can be somewhat modified by the donor myotubes. Although several groups have postulated the existence of fibre type - specific trophic factors (Funakoshi *et al.* 1995; Thibault *et al.* 1981), this theory only extends as far as fast vs. slow myotubes, and does not delineate between the various populations of fast motoneurons. The possibility that there are limited trophic factors causing the interaction of fast motoneurons with fast myotubes, and that the nerve is modulated after to correspond with the more specific fast fibre type, seems more likely. The known existence of only two muscle specific motoneurons, fast or slow, also indicates that this may be the case. Both of these scenarios suggest that the homotypic myotubes are exerting some kind of influence over the nerve, and that this influence is inherent to the myoblast population, itself.

A model describing muscle fibres as exhibiting a range of potential phenotypes has been hypothesized by Westgaard and Lomo (1988), who observed differences in contraction speeds from the EDL and soleus muscles when presented with identical electrical stimulation patterns. This adaptive range model suggests that myogenic lineages exist which have "different but overlapping sets of genes available to them." A similar model has been proposed by Hoh and Hughes (1989) who observed differences between muscles located in the jaw and muscles located in the hindlimb of cats. Hindlimb muscles transplanted into the jaw could exhibit a faster phenotype, but the fibres were never observed to express the superfast MyHC found normally in the masseter muscle (Hoh and Hughes, 1988). The term "allotype" was used to describe the range of possible phenotypes a particular myoblast population could obtain. Environmental influences could alter the phenotype of the resulting muscle fibres, but only within the allotype's range. The studies presented here detected innervation based on the localization of NCAM. During muscle development, NCAM can be localized to the entire surface of the myotube (Covault and Sanes, 1986), however, upon mature innervation, these proteins become specifically localized to the peri- and subsynaptic membrane (Covault and Sanes, 1985). Lesion of the nerve by crush injury causes a re-expression of NCAM along

the entire surface of the fibres (Covault *et al.* 1986), as does regeneration of muscle fibres (Irintchev *et al.* 1994). Upon reinnervation, the NCAM once again becomes localized to the synaptic site (Covault and Sanes, 1985). Therefore, the presence of NCAM along the cell surface indicates that mature innervation has not been attained.

The presence of cell accumulations in the area of the injection site is also of interest. Previous studies suggested that myoblast injections with cell lines form tumours in some instances (Wernig *et al.* 1991). Our findings here indicate that the accumulations of cells at the injection sites are not L6-derived, but rather represent lymphocyte invasion associated with the use of cyclosporin. No ß-gal activity was observed in any tumour - like accumulation, and these cell growths did not express any MyHC isoforms, indicating that the cell accumulations were not due to uncontrolled muscle cell growth. It has previously been reported that cyclosporin, in the process of shutting down T-cells, may allow the formation of lymphoproliferative tissue (Thiru, 1989). This build-up is due to the continued antigenic stimulation of the muscle graft causing proliferation of B-lymphocytes in the area. Since there are no other areas of antigenic stimulation in the body, these cell growths should be confined to only the injected leg, which is the case. The infiltrating B-lymphocytes cannot target the foreign cells without the aid of T-cells, so they are ineffective in removing the graft. This accumulation of B- cells may become a lymphoma, but initially is only a lesion (Thiru, 1989).

In conclusion, L6BAG-A4 cells seem to maintain their *in vitro* expressional pattern of MyHC, even after innervation in several different muscle environments. Since it is possible that these myotubes may be exerting some control over their environment, it is necessary to examine their developmental potential after they are placed in an environment in which the innervating motoneurons would not normally support the IIX phenotype. To do this, heterotypic fibres will be examined, and the fate of these cells will be determined in individual fast IIB, IIA, and slow muscle fibres.

CHAPTER 7- DEVELOPMENTAL POTENTIAL OF INJECTED L6BAG-A4 MYOBLASTS FOLLOWING INCORPORATION INTO HOST MUSCLE FIBRES

7.1 Introduction

The maintenance of the L6 *in vitro* phenotype in homotypic L6BAG-A4 myotubes suggests that these cells represent a distinct lineage of myoblasts destined to become IIX fibres. The lack of any correlation between their state of innervation and their MyHC expression further suggests that their developmental potential is established prior to innervation. However, since it is impossible to characterize the nerves that are innervating these myotubes (ie. slow versus fast), it is not possible to conclude that the intrinsic program will override any type of innervation. To properly address this question, the fate of L6BAG-A4 myoblasts was examined following incorporation into various host muscle fibre types to form heterotypic muscle fibres.

Analysis of heterotypic myotubes with nuclei from different origins *in vitro* has previously demonstrated perinuclear accumulations of some proteins (Piette *et al.* 1993; Pavlath *et al.* 1989) and all mRNA transcripts (Ralston and Hall, 1992; Piette *et al.* 1992). In the case of protein nuclear domains, it appears that the domains are a result of proteins being associated with a target (Hall and Ralston, 1989). Nuclear domains may be associated with membrane specializations, such as the acetylcholine receptor protein (Simon *et al.* 1992) which resides within a small area adjacent to the nuclei in the motor endplate region, or represent the integration of structural proteins into the cytoskeleton near the nuclei from which their mRNA are produced (Hall and Ralston, 1989). Previous studies have shown that such an organization is found for MyHC isoforms within heterokaryons established *in vitro* between human and mouse myoblasts (Pavlath *et al.* 1989).

To study the effects on MyHC expression of myoblasts *in vivo*, myoblasts were placed into foreign muscle environments using the technique of myoblast

transplantation. This technique was developed using mdx mice, with the ultimate aim of introducing normal myoblast nuclei into the limbs of patients with genetic disorders specifically affecting muscle tissue (Law, 1992; Karpati, 1991; Karpati, 1992), but has also been used to answer more basic scientific questions. Upon injection of donor myoblasts, two possible types of fusion may occur. Fusion of donor cells to host cells can result in heterotypic fibres, in which the different nuclei share the same cytoplasm. Alternatively, donor myoblasts may only fuse with themselves, resulting in homotypic fibres which only contain the nuclei of one cell population. These homotypic fibres have previously been reported in studies using a rat myoblast cell line (Chapter 6), and by Stockdale's group(DiMario *et al.* 1993; DiMario and Stockdale, 1995), following injection of primary quail myoblasts of both fast and slow origin into the hindlimb buds of 5 day old chick embryos. Both studies showed a complete maintenance of MyHC expression characteristic of the donor cells regardless of which muscle they were injected into. Another study, which analyzed the fate of heterotypic fibres using the same method and either a mouse myoblast cell line (C2C12) or primary mouse satellite cells as the donor cells showed very different results. Clones of myoblasts from either fast or slow muscles were injected into mouse muscles and their fibre-type potential analyzed 38 days post-injection. These experiments showed that injected cells lost their *in vitro* phenotype and adopted the phenotype of the fibres that they fused with (Hughes and Blau, 1992).

To examine the question of whether the fate of myoblast nuclei is controlled by internal genetic programs or external influences, such as innervation, L6 rat myoblasts infected with a constitutive, ß-galactosidase (ß-gal) reporter gene were injected into regenerating hindlimb muscles of immunosuppressed, adult rats and their fibre-forming potential examined immunohistochemically over an 8 week period using a panel of antibodies specific for the different myosin heavy chain (MyHC) isoforms. In particular, muscle fibres containing donor nuclei and exhibiting mature muscle characteristics were analyzed for their MyHC expression.

Interestingly, when heterotypic fibres were examined in predominantly type IIB (superficial tibialis anterior) and mixed type II (gastrocnemius) muscles, the IIX phenotype of the L6 myoblasts was observed, often in conjunction with the normal MyHC isoform. However, upon examination of individual muscle fibres in typically slow muscles, the IIX phenotype was only transiently expressed, and by eight weeks, heterotypic fibres expressing both slow and type IIX MyHCs could not be detected. These findings demonstrate that fast muscle fibre types represent a permissive environment for the expression of IIX MyHC by L6-derived nuclei. However, incorporation of L6 myoblast nuclei into slow muscle fibres represses the expression of IIX MyHC. This suggests that, while slow motoneurons and /or muscle cytoplasm can override the intrinsic program of L6 myoblasts, fast IIA and IIB motoneurons and/or muscle cytoplasm do not alter the fate of L6 nuclei.

7.2 Materials and Methods

7.2.1 Injection of L6 Myoblasts into Regenerating Adult Muscles

The muscle sections that provided the experimental material for this study were obtained from the injection series described in section 6.2.2. Following sectioning of the injection sites, slides were analyzed using X-gal immunohistochemistry as described in Chapter 6. Injection sites that showed putative heterotypic fibres containing both donor and host nuclei were chosen for further analysis.

7.2.2 Indirect Immunohistochemical Analysis of MyHC Expression in L6BAG-A4-Derived Muscle Fibres

Once injection sites containing heterotypic fibres were identified based on X-gal immunohistochemistry, serial sections of the injection sites were fixed with 90% methanol for 6 min at -20°C, blocked for 30 min with 10% goat serum (Cedarlane Labs Ltd, Hornby, Ont) in phosphate buffered saline (PBS) at 37°C and then

incubated with the various MyHC-specific antibodies for 1 hr at RT. The monoclonal antibodies used for these characterizations included 47A, MY-32, 4A.74, 212F, and 8H8. The specificity, optimal dilution and isotype of these antibodies have previously been described (Table 2.1). Following incubation in the primary antibodies for 1 hr at RT, sections were rinsed several times with PBS and incubated in a 1:50 dilution of fluoroscein (FITC) or rhodamine (RITC)-conjugated rat anti-mouse (RAM) IgG secondary antibody (ICN Biomedicals Canada Ltd, Mississauga, Ont) in bovine serum albumin (BSA; Gibco/BRL, Burlington, Ont)-PBS for 1 hr at RT. After a final rinse, sections were coverslipped with a 50% glycerol solution in PBS containing 5% paraphenyldiamine (both supplied by BDH Inc, Toronto, Ont) and 0.5% Hoescht dye (Sigma Chemical Co, St Louis, MO). In addition to these single localizations, double localizations, using two different Mabs specific for fast (MY-32) or slow (8H8) MyHCs, were performed as described in section 2.2.7. Results were photographed using a Zeiss Axiophot photomicroscope using Kodak T-MAX 400 film (commercially available).

7.2.3 ABC - Immunohistochemical Analysis of MYHC Expression in L6BAG-A4-Derived Muscle Fibres

Sections were obtained as described in the previous section and examined for MyHC expression using Avidin Biotin Complex (ABC)-fluorescence or ABC-alkaline phosphatase immunohistochemistry. Sections were blocked in 10% goat serum in PBS for 30 min at 37°C, incubated in primary antibody for 1 hr and washed with PBS. Primary monoclonal antibodies 47A (1:10), MY-32 (1:200), 212F (1:3), 4A.74 (undiluted), SC.71 (undiluted), BF.F3 (undiluted), BF.35 (undiluted), 8H8 (1:50), 10D10 (1:5), 4A9 (1:50), and 4A.951 (undiluted) were used for these analyses. In addition, rabbit polyclonal antibody NN6 (1:350) was used, which specifically recognizes neonatal MyHC. Sections were then incubated with a secondary antibody; a 1:1000 dilution of either biotinylated goat anti-rabbit (GAR) IgG (against the NN6 antibody) or rabbit anti-mouse (RAM) IgG_{2A} (against the 47A

antibody) for one hr (GAR IgG; Tago Inc, Burlingame, CA, RAM IgG; ICN Biomedicals, Mississauga, Ont). After the PBS washes, a rhodamine-conjugated avidin complex (at a dilution of 1 in 50) or a biotin-avidin complex (Dimension Labs, Mississauga, Ont), was placed on the sections for 1 hr. After washing, sections were coverslipped as previously mentioned. Colour pictures were taken using Kodacolour Gold 400 ASA colour print film (commercially available).

7.2.4 Determination of Fibre Types Based on Mab Labelling

Following ABC-AP immunolocalizations using MyHC - specific Mabs, sections were analysed for the number of host/donor heterotypic fibres (which stained for X-gal and showed characteristics typical of mature fibres) and host derived mature fibres. To prevent the possibility of false positives and negatives, only fibres clearly belonging to one group or another were scored. Scoring of host derived fibres was limited to the area immediately adjacent to the injection site so that regional differences in the muscle could be minimized. To provide accurate counts, specific muscle fascicles were analyzed in each serial section. The number of positive fibres within these areas was determined after labelling with 212F (IIB/IIX), 4A.74 (IIA), SC.71 (IIA/IIX), BF.35 (all MyHCs except embryonic and IIX), BF.F3 (IIB) and 10D10 (slow). Fibres were then classified as types I, IIA, IIA/IIX, IIX, IIB/IIX or IIB. To determine the number of fibres belonging to each group the following equations were used:

(A) Total = # of X-gal positive fibres

(B) I (slow) = # of 10D10 positive fibres

(C) IIA = Total # of fibres (A) - # of 212F positive fibres - # of Type I fibres (B)

(D) IIA/IIX = # of SC.71 fibres - # of Type IIA fibres (C)

(E) IIX = # of 212F positive fibres - # of BF.F3 positive fibres - # of IIA/IIX fibres (D)

Since 212F recognizes both IIX and IIB, it is impossible to accurately determine the number of IIB/IIX fibres. However, Mab 4A.74 has been shown to consistently cross-react with IIX MyHC at higher levels of expression. Therefore, it is possible to obtain a lower limit to the number of fibres containing both IIX and IIB MyHC using the equation:

IIB/IIX = # of 4A.74 positive fibres - # of IIA fibres - # of IIA/IIX fibres - # of IIX fibres

Therefore, the number of IIB fibres can be determined by :

IIB = # of BF.F3 positive fibres - # of IIB/IIX fibres

From these equations the percentage of fibres belonging to each class was determined for muscle within and outside of the grafts.

7.3 Results

To study the effects of different environments on the maintenance of the L6 phenotype, several muscles were targeted for injection. The muscles that were targeted included the gastrocnemius and tibialis anterior, both of which exhibit predominantly fast and predominantly slow areas of muscle fibres, the plantaris, which is a mixed muscle, and the soleus, which is typically slow (Armstrong and Phelps, 1984). Therefore all of the muscles contain areas of fibres that are not similar to L6 myotubes in MyHC expression, and, therefore, represent foreign environments in which the maintenance of the L6 phenotype may be most apparent. X-gal fibres exhibiting mature muscle fibre characteristics were observed as early as two weeks after injection. Analysis of the MyHC profile of these fibres, which were believed to contain both donor and host nuclei, revealed patterns of MyHC expression similar to L6 cells *in vitro* when found in typically fast muscles. However, similar localizations in typically slow area showed only a transient expression of the L6 *in vitro* phenotype.

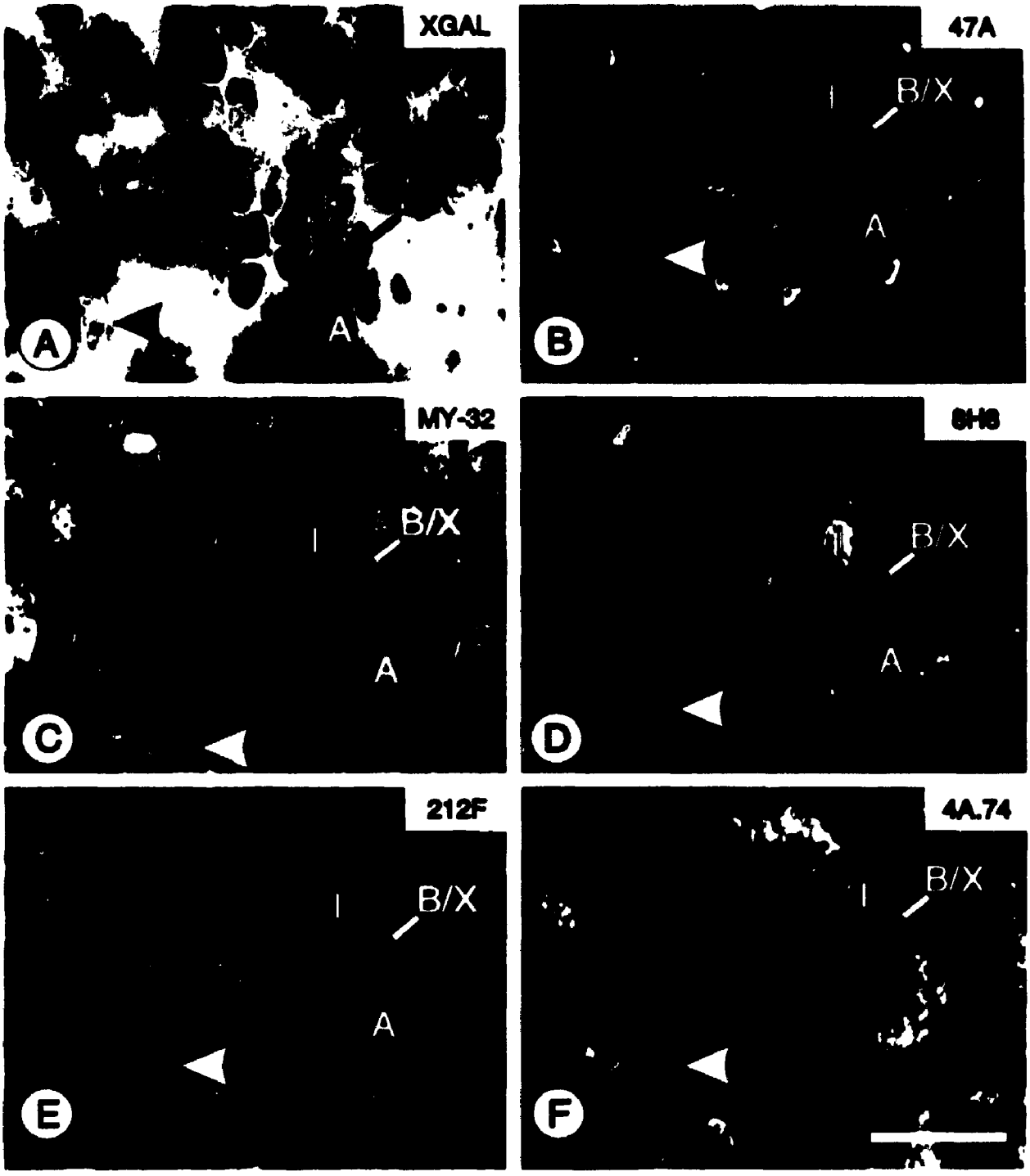
7.3.1 Characteristics of Heterotypic Fibres

Following injection, L6 myoblasts formed both homotypic and heterotypic fibres. Homotypic fibres (see Chapter 6) resulted from the fusion of donor cells with each other to form new myotubes which showed intense staining with X-gal and centrally located nuclei characteristic of regenerating fibres (Benoit and Belt, 1970). Heterotypic fibres, which were the result of donor cells fusing to host myoblasts or host fibres, were very rare one week after injection. However, at two weeks after injection, they were quite numerous, and could be found in all muscles examined. At times, they appeared several hundred micrometres away from the actual injection site. These fibres showed markedly different characteristics when compared to homotypic fibre, exhibiting lighter intensities of X-gal labelling, the presence of peripherally-located nuclei and a polygonal-shape in cross section typical of mature muscle. The difference in X-gal staining is most likely due to the lower ratio of

donor nuclei to host nuclei in the fibres. In addition, X-gal analysis on longitudinal sections, or on cross sections several hundred microns away from the injection site, revealed changes in the X-gal labelling, with some areas not being labelled. These fibres also have much larger diameters and are separated from adjacent fibres by only a small amount of connective tissue - the endomysium. These characteristics indicate that the fibres are mature and, therefore, are governed by environmental influences similar to the surrounding muscle. Heterotypic fibres did not stain upon characterization with a polyclonal antibody specific for NCAM, indicating that they are fully innervated (data not shown).

Four weeks after injection, large areas of heterotypic fibres could be found in many of the muscles injected. After X-gal staining and MyHC characterization of an injected plantaris muscle, it was determined that the L6 cells did not show a particular pattern of fusion (Figure 7.1). Donor cells could be detected in all fibre types indicating that the formation of heterotypic fibres does not involve recognition of L6 cells by a particular subset of host satellite cells or myotubes (representing a fibre type group). MyHC characterization showed X-gal positive fibres staining with MY-32 (all fast MyHCs), 8H8 (slow MyHC), 4A.74 (IIA), and 212F (IIB/IIX) representing all possible types of fibres based on MyHC content. These findings suggest that L6 myoblasts fuse promiscuously with the host satellite cells and myotubes that will go on to form all of the different fibre types. Since labelling with Mab 4A.74 should not recognize IIX fibres in this experiment because of the lower level of sensitivity inherent in indirect immunohistochemistry used here, Mabs 4A.74 and 212F should stain mutually exclusive populations of fast fibre. However, fibres labelled for both 4A.74 and 212F were observed, suggesting that these fibres are expressing two different adult fast MyHCs. The expression of two MyHC isoforms was rarely observed in the contralateral limbs, and were strictly confined to IIA / slow expressing type IIC fibres only (see section 6.3.1).

Figure 7.1 L6 cells fuse with both fast and slow host muscle fibre types during muscle regeneration. Serial sections of rat plantaris muscle four weeks post-injection were analyzed for ß-gal expression using X-gal substrate (A) and for MyHC expression using immunofluorescence with Mabs 47A (B), MY-32 (C), 8H8 (D), 4A.74 (E) and 212F (F). Primary Mabs were recognized by a fluorescein conjugated secondary antibody. Mab 47A (B) reacts specifically against embryonic MyHC normally expressed by L6 myotubes *in vitro*, and can be detected in small, X-gal positive fibres (*). Mab 8H8 (D) reacts against slow type I MyHC while the adult fast isoforms IIA, IIB and IIX are recognized by Mabs 212F (F; IIB and IIX) and 4A.74 (E; IIA) and MY-32 (F; IIA,IIB and IIX). A comparison of X-gal staining with MyHC expression reveals that L6-BAG A4 cells can be found in every possible fibre type. Bar = 55 µm.


XGAL
A
A
47A
B/X
A
B
MY-32
B/X
A
C
6H3
B/X
A
D
212F
B/X
A
E
4A.74
B/X
F

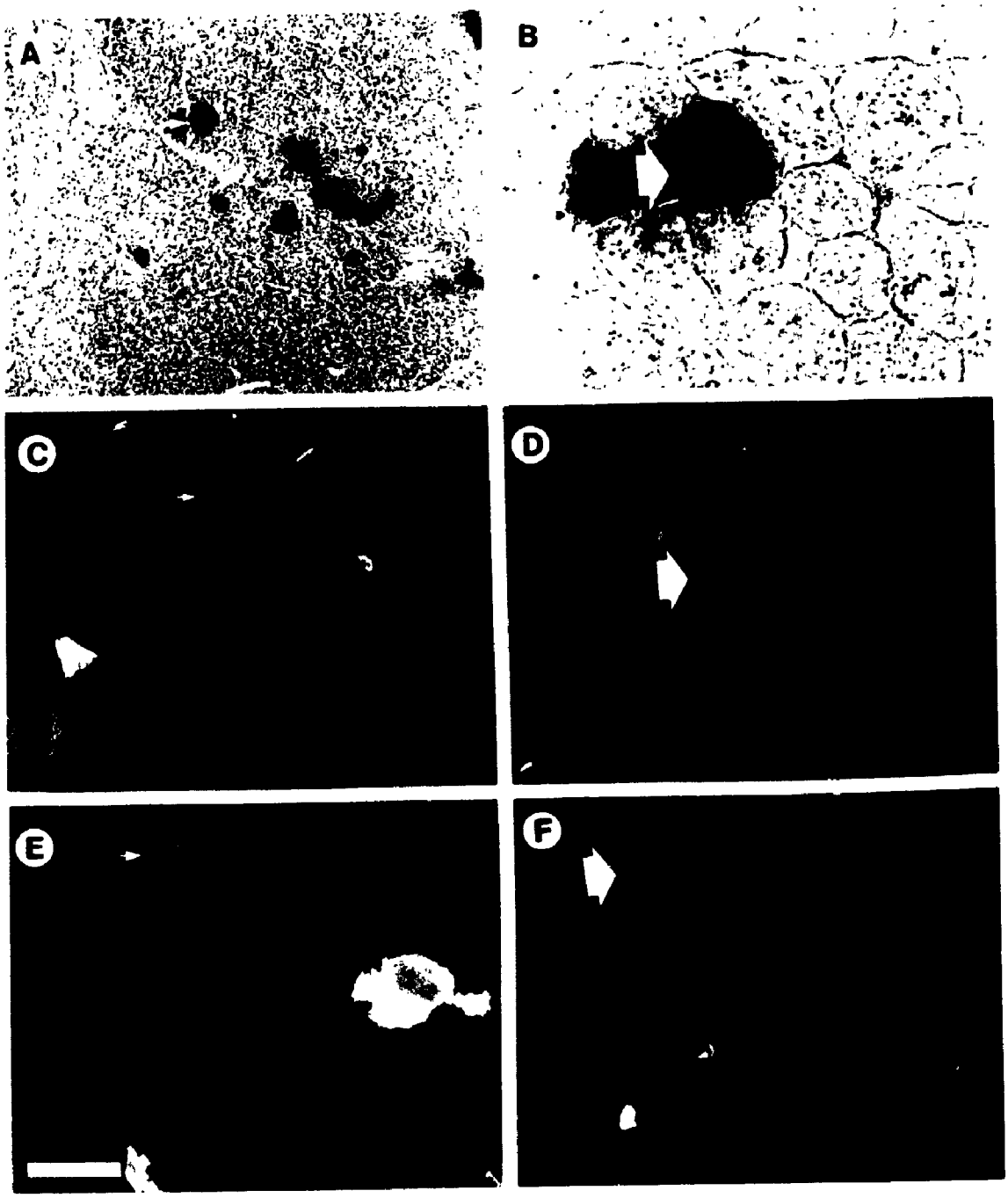
7.3.2 Expression of Embryonic MyHC in Heterotypic Fibres After Injection into the Tibialis Anterior Muscle

To determine if the *in vitro* phenotype of L6 myoblasts was maintained when donor and host nuclei were present in a common cytoplasm, heterotypic fibres were first examined for the expression of embryonic MyHC. Immunolocalization of embryonic MyHC was carried out on the tibialis anterior muscle since this represents a mixed muscle. Characterization of heterotypic fibres using ABC-AP immunolocalization of 47A revealed mature fibres that expressed embryonic MyHC in a regionalized manner around individual nuclei beginning at two weeks. Further analysis indicated that similar nuclear domains could be observed up to eight weeks after injection, and that the expression of these domains was restricted to fast fibres only.

Upon characterization of an experimentally-injected tibialis anterior 14 days after myobast transplantation, putative heterotypic fibres were observed up to several hundred microns away from the injection site in areas of the muscle that exhibited no signs of regeneration (such as centrally-located nuclei and neonatal MyHC expression, Figure 7.2). These observations indicate the great migratory ability of the cells and also suggest that donor cells may be fusing onto partially degenerated fibres or even mature uninjured fibres. By characterizing these fibres using ABC-fluorescent localization with MyHC-specific antibodies, it can be seen that several of them express embryonic MyHC in a regionalized manner. These accumulations are concentrated around individual nuclei in one area of the fibre and lightly distributed throughout the rest of the cross-sectional area of the fibre. The fibres appear to be mature fibres since they are not labelled by NN6 which specifically recognizes neonatal MyHC, characteristic of regenerating fibres. Also, the expression of embryonic MyHC seems to be restricted to fast heterotypic fibres, since no fibres exhibiting nuclear domains were labelled with 10D10 (data not shown).

To ascertain if the expression of embryonic MyHC is transient, and not due

Figure 7.2 Rat tibialis anterior muscle two weeks after injection showing a putative nuclear domain of embryonic myosin in a fully mature, heterotypic muscle fibre viewed at low (A,C,E) or high (B,D,F) magnification. Serial sections were characterized for X-gal staining (A,B) or MyHC expression using ABC -fluorescence using Mabs against embryonic MyHC (Mab 47A; C and D) or neonatal MyHC (NN6; E and F), and primary antibodies were identified with rhodamine conjugated secondary antibody. X-gal staining is fairly uniform (A and B) and shows donor cell fusion to two adjacent fibres (▸). One of the fibres maintains some degree of embryonic MyHC expression localized mainly to the periphery of the fibre (C and D), with some lighter staining throughout the rest of the fibre. The absence of neonatal MyHC (E and F), along with peripheral nucleation revealed by Hoescht dye staining (E), suggests that this fibre is fully mature and, under normal circumstances, would not express embryonic MyHC. Bar = 100 μm (A,C,E) or 40 μm (B,D.F).


A
B
C
D
E
F

to the effects of muscle regeneration, heterotypic fibres were analyzed at both six and eight weeks after myoblast transplantation into the tibialis anterior (Figure 7.3). ABC-AP localization of Mab 47A revealed the persistence of embryonic MyHC in heterotypic fibres at these later time points. Similar localizations on the contralateral limbs revealed a complete absence of any embryonic MyHC expression (Chapter 6, Figure 6.4). Once again these nuclear domains were observed in MY-32 positive fibres only, which suggested that the regional expression of embryonic MyHC is restricted to fast fibre types only (data not shown). To determine the approximate length of these nuclear domains, longitudinal sections from the tibialis anterior eight weeks after injection were characterized. Nuclear domains of embryonic MyHC were seen within adult fast type fibres. These accumulations contained a centrally located nucleus and spread approximately 20 - 25 μm in either direction. The length of the nuclear domains was determined by measuring the boundaries of the intense staining. These results suggest that the embryonic MyHC is expressed after fusion to fast muscle fibres in the host. Whether this expression is due to the continued fusion of L6BAG-A4 myoblasts throughout the experiment or due to a maintenance of the *in vitro* phenotypic profile of these cells could not be determined.

7.3.3 Expression of the IIX MyHC Isoform in Heterotypic Fast Muscle Fibres

Although the embryonic MyHC isoform is observed in putative heterotypic fibres throughout the course of the experiment, its absence in some X-gal staining fibres suggests that the expression is transient. Since this isoform is replaced by the IIX MyHC in homotypic myotubes (Chapter 6), it was necessary to determine the expression of this isoform in donor and host derived fibres. Characterization of muscles that contained either predominantly IIB or mixed fast fibre types revealed a maintenance of the IIX phenotype, often in conjunction with other fast isoforms.

To analyze the fate of the IIX MyHC isoform in fast heterotypic muscle fibres, ABC-AP immunolocalizations were used to characterize injection sites in the tibialis anterior muscle six weeks after injection (Figure 7.4). The tibialis anterior

Figure 7.3 Nuclear domains of embryonic MyHC persist in adult fast heterotypic fibres after injection of L6 myoblasts into tibialis anterior. X-gal immunohistochemistry revealed large areas of heterotypic fibres in the tibialis anterior at 42 (A) and 56 (B) days after injection. Higher magnification of the boxed areas show ABC - AP immunohistochemistry of 47A revealing nuclear domains of embryonic MyHC within adult fast fibres in the tibialis anterior at 42 (B,E) and 56 (C,F) days after injection. These domains surround a single nuclei (►) and range between 40 and 70 µm in length. These fibres also react with MY-32 (data not shown) indicating that they have an adult fast phenotype. Homotypic fibres between the larger fibres can also be seen (→). Bar = 500 µm for A and B, 40 µm for C and E, and 80 µm for D and F.

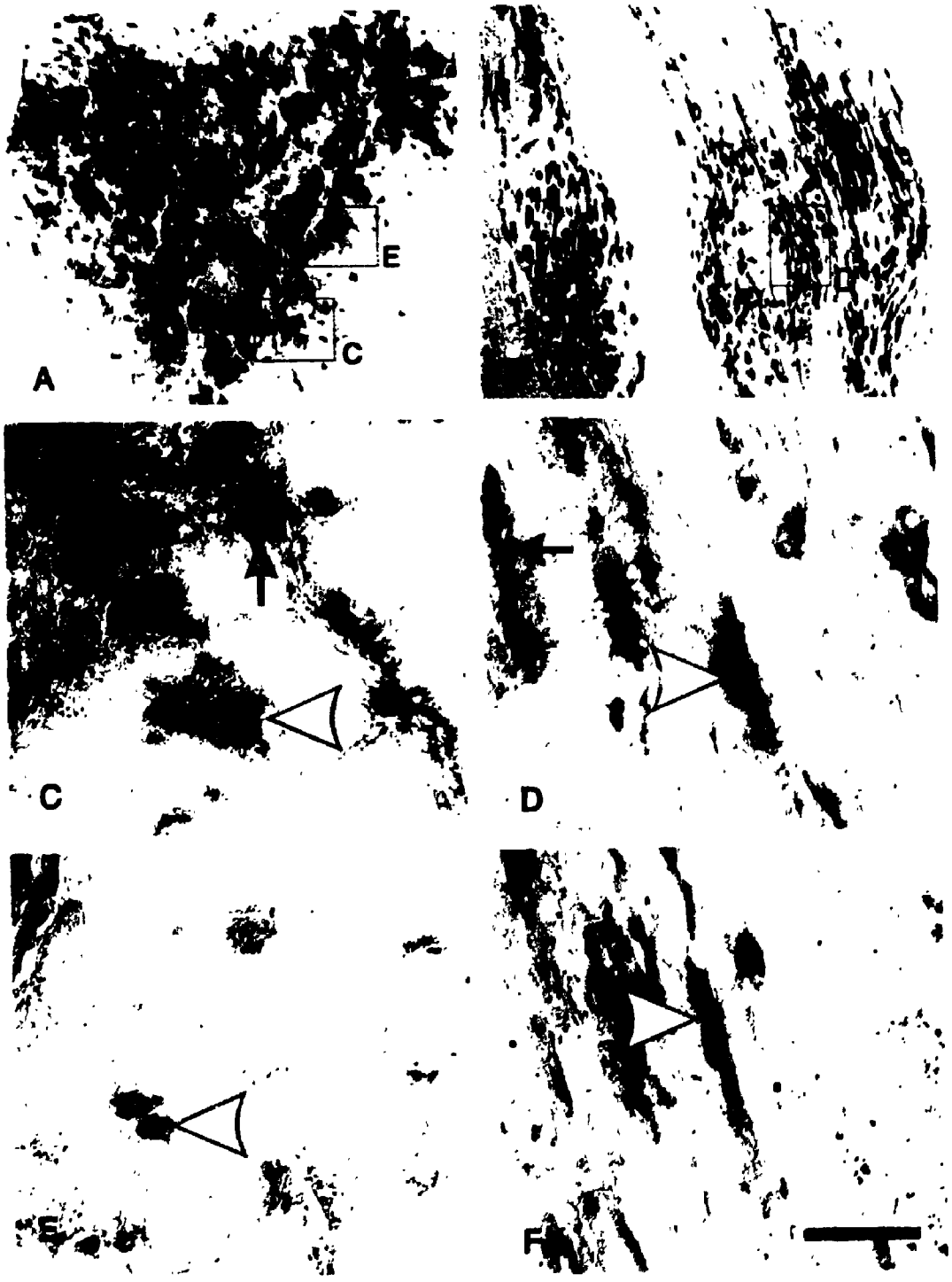
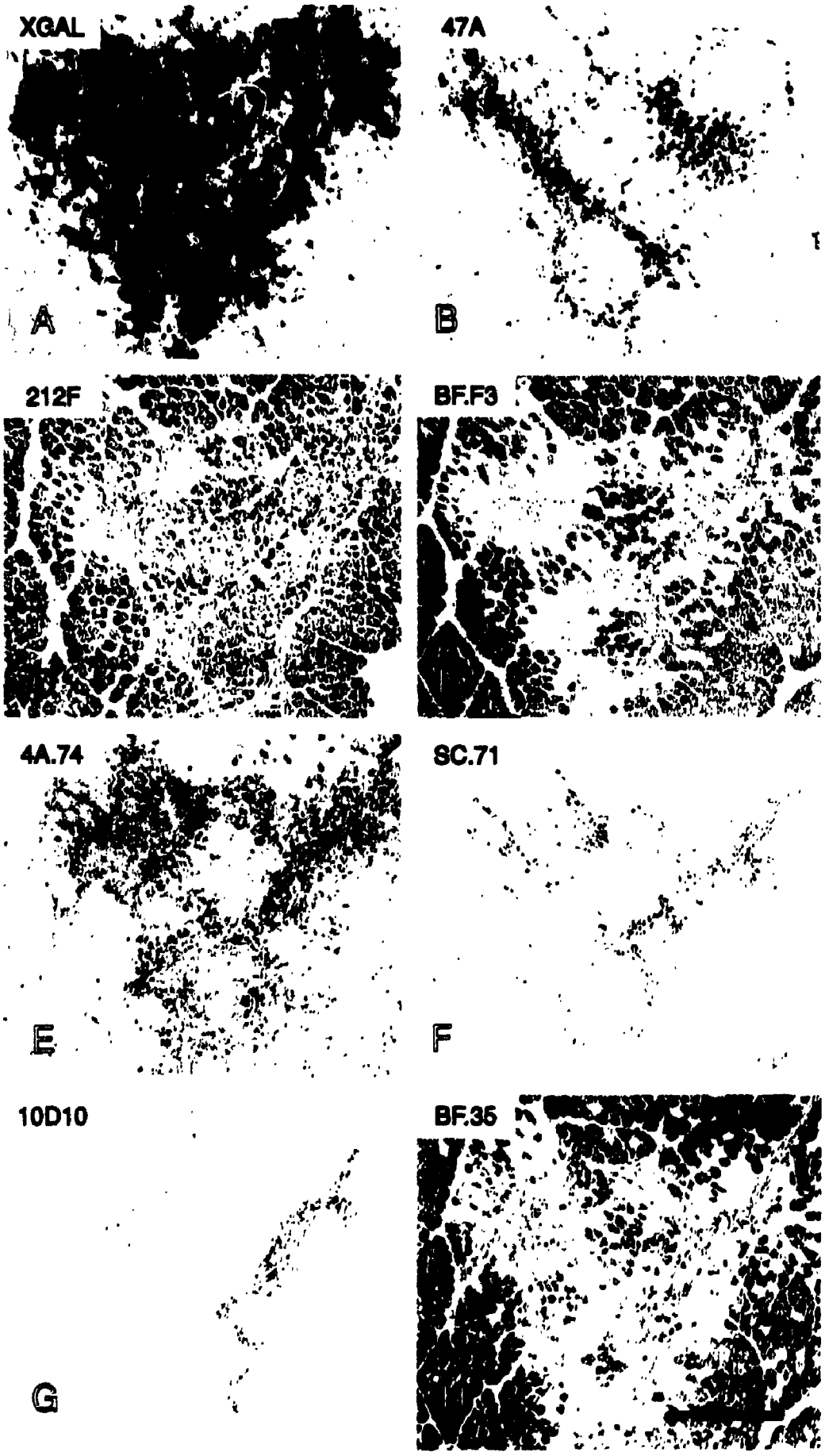

A
E
C
C
D
E
F

Figure 7.4 Characterization of heterotypic fibres 42 days after injection of L6 myoblasts into the tibialis anterior of adult Wistar Furth rats. Serial sections were either stained for X-gal (A) or analyzed with ABC-AP immunolocalizations for MyHC - specific Mabs for embryonic (47A;B), fast IIB/IIX (212F;C), fast IIB (BF.F3;D), fast IIA (4A.74, which cross-reacts with IIX; E and SC.71;F), slow (10D10;G), and all isoforms except IIX and embryonic (BF.35;H). A large area of heterotypic, X-gal stained fibres (A) can be seen to react predominantly with 212F (C) and not with BF.35 (H) indicating the presence of a large population of IIX fibres in a area of the muscle that is predominantly IIB in phenotype (see accompanying table). No other Mab stains to the same extent as 212F, however 4A.74 (E) shows cross-reaction to this area supporting the idea that this is a predominantly IIX area. Bar = 575 µm.


XGAL
A
47A
B
212F
BF.F3
4A.74
E
SC.71
F
10D10
G
BF.35

muscle contains a superficial region which is predominantly IIB in normal adult rats (Armstrong and Phelps, 1984). X-gal immunohistochemistry revealed a large injection site containing large numbers of putative heterotypic fibres based on cross-sectional shape, peripheral nucleation and decreased intensity of X-gal labelling, present within the superfical region. Upon characterization with Mabs specific for embryonic MyHC (47A), fast IIX/IIB MyHCs (212F), fast IIA MyHC (SC.71 and 4A.74), fast IIB MyHC (BF.F3), slow MyHC (10D10) and all isoforms except IIX and embryonic (BF.35), it was observed that a large proportion of these fibres were not recognized by BF.35, but were recognized by 212F. This suggested that these fibres expressed predominantly IIX MyHC. Further characterization of this graft with SC.71 revealed a complete absence of the IIA isoform. Interestingly, 4A.74 appeared to lightly label the majority of these fibres. This confirmed the presence of the IIX isoform in these fibres since this Mab has been shown to consistently cross-react to high levels of the IIX MyHC (section 2.2.1). To determine the specific expression of each of these fibres, as well as compare the proportions to areas outside the graft, specific fascicles containing either all X-gal positive fibre or all X-gal negative fibres were identified and scored for MyHC expression labelling. This data was used to calculate specific proportions of each fibre type including types IIB, IIB/IIX and IIX (Table 7.1). Types I and IIA fibres were within or adjacent to the graft and, therefore, did not enter into the calculations. Analysis revealed that 47.4% of the heterotypic fibre stained exclusively for the IIX MyHC, an increase of almost 40% from outside the graft. In addition, another 21.6% of the fibres expressed both fast IIB and IIX, a phenomenon observed only rarely (0.5%) outside the graft, or in contralateral limbs (data not shown). Therefore, 69% of the fibres expressed IIX MyHC, an increase of 61.3% from the proportion of IIX fibres outside the graft. In contrast, the proportion of exclusively IIB fibres dropped from 90.8%, which represented areas completely surrounding the graft, to 31% within the graft. These results suggest that the L6 nuclei present within these IIB fibres are maintaining their IIX phenotype.

Table 7.1A Myosin heavy chain expression of β-galactosidase positive fibres in the tibialis anterior after injection of L6BAG-A4 myoblasts

Monoclonal Antibody	# of Fibres Within I.S.	# of Fibres Outside I.S.
212F	329	194
BF.35	120	177
BF.F3	173	176
4A.74	227	16
SC.71	0	1
10D10	0	0
Total Counted	329	195

Table 7.1B Fibre types in the tibialis anterior after injection of L6BAG-A4 myoblasts

Fibre Type	Within I.S.	% of Total Fibres	Outside of I.S.	% of Total Fibres
IIX	156	47.4	16	8.2
IIB	102	31	177	90.8
IIA	0	0	1	0.5
IIX/IIB	71	21.6	0	0
IIA/IIX	0	0	0	0
I	0	0	0	0

A similar characterization of heterotypic fibres was carried out in an area of the gastrocnemius that contained all fast fibre types (Figure 7.5). Characterization of the muscle with X-gal immunohistochemistry once again revealed the presence of a large injection site that contained many heterotypic fibres. ABC-AP immunolocalization with the various MyHC-specific Mabs revealed that these fibres were uniformly recognized by Mab 212F. Further examination indicated that these fibres expressed the IIX MyHC isoform, since neither BF.F3 or BF.35 labelled the majority of the fibres. Evaluation of the MyHC expression of these fibre (Table 7.2) revealed that almost 60% of the fibres expressed exclusively IIX MyHC, and that another 7% expressed the IIX isoform in conjunction with another fast isoform. Therefore, the total proportion of IIX expressing heterotypic fibres is 66.8%, an increase of 53.3% over regions outside of the graft. In contrast, the proportion of exclusively IIA fibres dropped from 9.3% to 1.8%, while exclusively IIB fibres decreased from 77.2% to 31.4%. These numbers indicate that the phenotypic expression of L6BAG-A4 nuclei is maintained in host/donor heterotypic fibres.

7.3.4 Expression of the IIX MyHC Isoform in Heterotypic Slow Muscle Fibres

The maintenance of IIX MyHC expression in heterotypic fibres in exclusively fast areas suggests that the environmental factors present did not affect the expression of the L6BAG-A4 nuclei. However, the environment may not represent a vastly different environment since there is no evidence to suggest that separate fast fibre populations are innervated by different classes of fast motoneurons.

To determine if the L6 BAG-A4 *in vitro* phenotype is maintained in a vastly different environment, myoblasts were transplanted into the red (medial) gastrocnemius, which represents a predominantly slow region of the muscle (Armstrong and Phelps, 1984). Analysis of the an injection site in the red gastrocnemius revealed a small group of X-gal positive fibres at the periphery of the muscle (Figure 7.6). The light intensity of X-gal labelling coupled with peripherally-located nuclei indicated that these were heterotypic fibres containing both host and

Figure 7.5 Characterization of heterotypic fibres 42 days after injection of L6 myoblasts into the gastrocnemius of adult Wistar Furth rats. Serial sections were either stained for X-gal (A) or analyzed with ABC-AP immunolocalizations for MyHC - specific Mabs for embryonic (47A;B), fast IIB/IIX (212F;C), fast IIB (BF.F3;D), fast IIA (4A.74, which cross-reacts with IIX; E and SC.71;F), slow (10D10,G), and all isoforms except IIX and embryonic (BF.35;H). A large area of heterotypic, X-gal stained fibres (A) can be seen to react predominantly with 212F (B) and not with BF.35 (H) indicating the presence of a large population of IIX fibres in a area of the muscle that is of mixed adult fast phenotype (see accompanying table). Once again 4A.74 (E) shows cross reaction to the area of X-gal staining that is not apparent for SC.71 (F), supporting the suggestion that this is a predomantly IIX area. Bar = 470 μm.

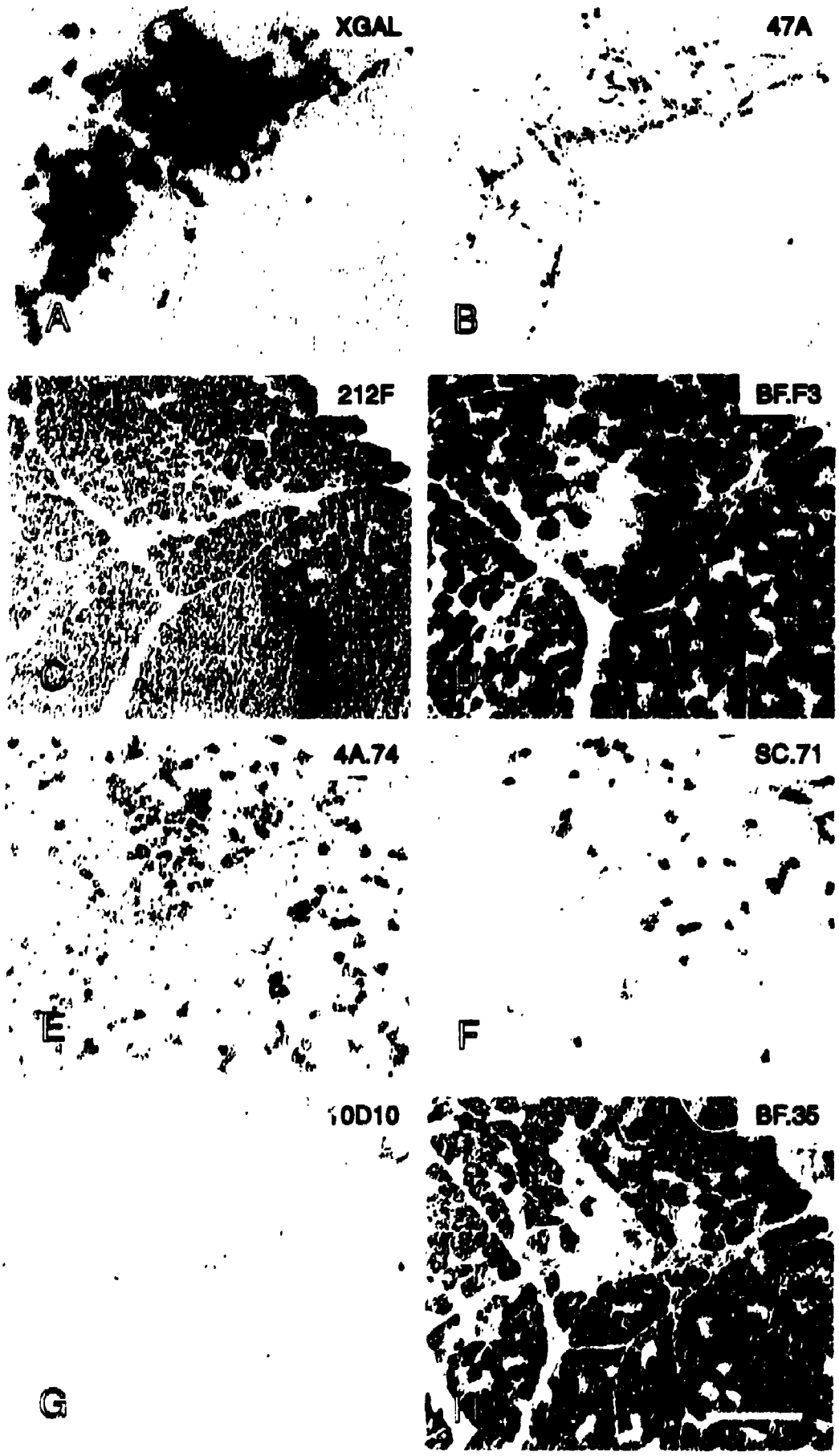

XGAL
A
47A
B
212F
C
BF.F3
4A.74
E
SC.71
F
10D10
G
BF.35

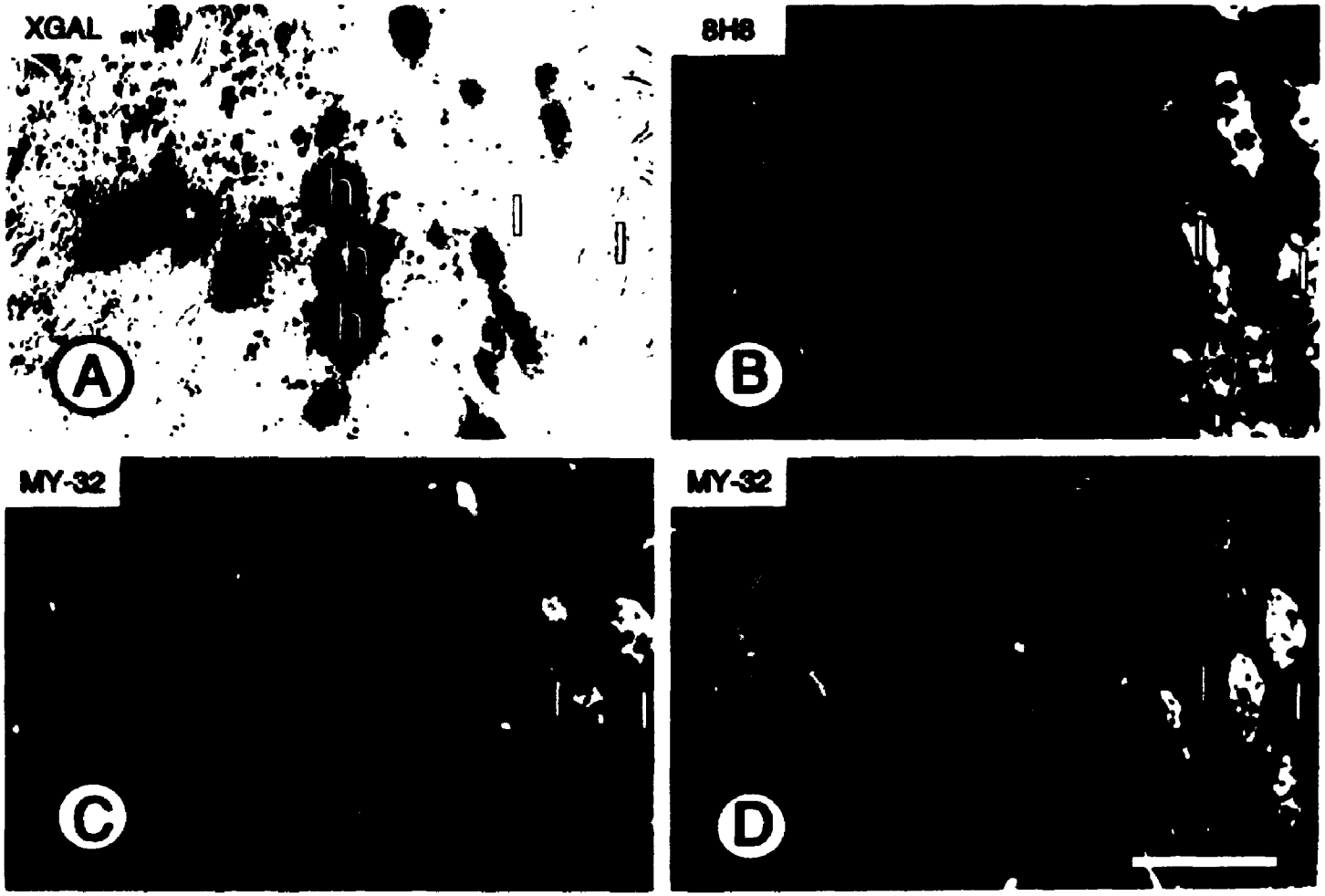
Table 7.2A Myosin heavy chain expression of β-galactosidase positive fibres in the gastrocnemius after injection of L6BAG-A4 myoblasts

Monoclonal Antibody	# of Fibres Within I.S.	# of Fibres Outside I.S.
212F	166	174
BF.35	67	168
BF.F3	61	149
4A.74	113	44
SC.71	7	19
10L10	0	0
Total Counted	169	193

Table 7.2B Fibre types in the gastrocnemious after injection of L6BAG-A4 myoblasts

Fibre Type	Within I.S.	% of Total Fibres	Outside of I.S.	% of Total Fibres
IIX	101	59.8	25	13
IIB	53	31.4	149	77.2
IIA	3	1.8	18	9.3
IIX/IIB	8	4.6	0	0
IIA/IIX	4	2.4	1	0.5
I	0	0	0	0

Figure 7.6 MyHC expression in the red gastrocnemius muscle four weeks after injection showing co-expression of slow and fast MyHC isoforms in ß-gal positive fibres. Serial sections from the injection site were analyzed for ß-galactosidase expression using X-gal substrate (A) or with immunofluorescence against slow (8H8;B) or neonatal/adult fast (MY-32;C,D) MyHC. Primary antibodies were recognized by isotype - specific secondary antibodies conjugated to fluorescein (8H8) or rhodamine (MY-32) After four weeks, both homotypic (h) and heterotypic (*) fibres can be seen based on morphology and the intensity of X-gal staining (A). Immunofluorescence reveals that the majority of homotypic fibres express adult fast MyHC (C,D) and not slow (B). The heterotypic fibres show co-expression of both adult fast and slow MyHCs (compare B and D), while normal host muscle fibres express only one of the two isoforms. Expression of the adult fast isoform is regionalized since sections 70 µm away did not show this pattern of staining of MY-32 staining (C). Bar = 33 µm.


XGAL
A
8H8
B
MY-32
C
MY-32
D

donor nuclei unlike the homotypic fibres found outside the muscle bed. Upon fluorescent localization of MyHCs within the graft, all of the observed heterotypic fibres were recognized by Mabs specific for both fast (MY-32) and slow (8H8) MyHCs. All of the fibres adjacent to the graft labelled for either MY-32 or 8H8 but not both, similar to the contralateral limbs (see Chapter 6). No labelling was observed when the graft was characterized with 4A.74 or 47A (data not shown) indicating that the fast isoform was not IIA, and that embryonic MyHC was not present at this time. Characterization of the injection site 100 μm in either direction revealed an absence of the fast isoform, suggesting that this MyHC was localized to a specific region within the fibre, presumably where fusion of the L6BAG-A4 myoblasts had occurred.

To confirm the observation that the IIX MyHC isoform is expressed in heterotypic fibres expressing the slow MyHC, putative heterotypic fibres were individually examined at eight weeks after injection onto the deep region of the plantaris muscle. This area of the plantaris contains a mixture of fibre types. Upon analysis with X-gal immunohistochemistry, a large area of heterotypic fibres could be observed (Figure 7.7). ABC-AP immunolocalizations using MyHC-specific Mabs was subsequently used to calculate the percentage of each fibre type. Unfortunately, the percentage of IIX/IIB fibres could not be determined since the 4A.74 Mab did not cross-react with the IIX expressing fibres. However, it was still possible to calculate the proportions of types I, IIA, IIA/IIX and IIX fibres (Table 7.3). Even though the percentage of exclusively IIX fibres did not show a large increase within the graft, 19.2% of the fibres expressed both IIX and IIA MyHCs. The total number of fibres which expressed IIX MyHC alone or in combination with IIA was 77.6%, an increase of 35.7% over the area outside the injection site.

Upon examination of the injection site with 212F and 10D10, heterotypic fibres co-expressing slow and fast MyHC were not detected.. These findings were confirmed by similar observations in the soleus and red gastrocnemius muscles at eight weeks after injection (data not shown). Although it is possible that different

Figure 7.7 Characterization of heterotypic fibre 56 days after injection of L6 myoblasts into the plantaris of Wistar Furth rats. Serial sections were either stained for X-gal (A) or analyzed with ABC-AP immunolocalizations for MyHC - specific Mabs for fast IIB/IIX (212F;B), fast IIA (SC.71;C), slow (10D10;D), adult fats IIA (4A.74;E) or all isoforms except IIX and embryonic (BF.35;F) MyHCs. A large area of heterotypic, X-gal stained fibres (A) can be seen in a mixed muscle containing all fibre types. Although this is a mixed area, it can be seen that the predominant fibre type is fast IIX, both within and outside of the graft site, since BF.35 (F) stains less than half of the fibres. Staining with 10D10 reveals the presence of several slow type fibres within the graft (➤), only one of which stains shows some cross reaction to 212F (→), indicating that the adult slow isoform does not colocalize with fast IIB/IIX. Bar = 90 μm.

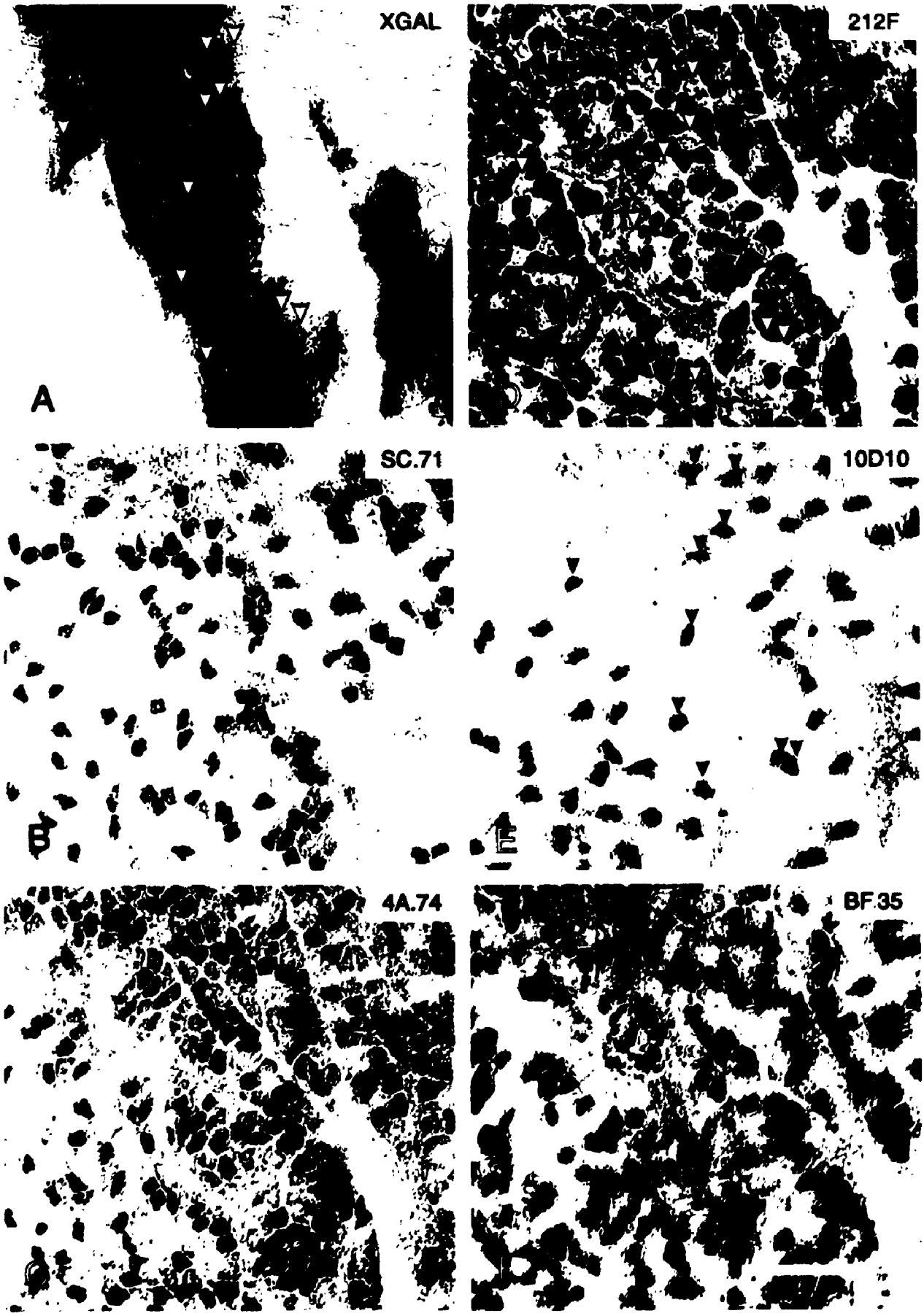

XGAL
212F
A
SC.71
10D10
B
4A.74
BF.35

Table 7.3 Myosin heavy chain expression of β-galactosidase positive fibres in the plantaris after injection of L6BAG-A4 myoblasts

Monoclonal Antibody	# of Fibres Within I.S.	# of Fibres Outside I.S.
212F	170	n.d.
BF.35	89	137
BF.F3	13	n.d.
4A.74	177	n.d.
SC.71	87	n.d.
10D10	13	n.d.
Total Counted	214	229

n.d - not determined

Table 7.3B Fibre types in the plantaris after injection of L6BAG-A4 myoblasts

Fibre Type	Within I.S.	% of Total Fibres	Outside of I.S.	% of Total Fibres
IIX	125	58.4	92	40.2
IIB	13	6.1	n.d.	n.d.
IIA	22	10.3	n.d	n.d.
IIX/IIB	0	0	n.d	n.d.
IIA/IIX	41	19.2	4	1.7
I	12	5.6	n.d	n.d.
I/IIX	1	0.4	n.d.	n.d.

n.d. - not determined

areas within a fibre may express one isoform or the other, these observations indicate that fusion of L6 myoblasts to slow fibres results in the down-regulation of IIX MyHC by L6 nuclei at eight weeks post injection.

7.4 Discussion

Muscle fibres resulting from the fusion of donor myoblasts to host satellite cells and myotubes have allowed us to examine the effects of a common cytoplasm on the expression of nuclei from different muscle precursor cell populations. When L6 myoblasts fused to typically fast host fibres, they maintained their expression of both embryonic and fast IIX MyHC. However, the incorporation of L6 nuclei into slow fibres resulted in transient expression of the IIX isoform only, and by eight weeks, IIX MyHC could no longer be observed in X-gal positive slow fibres. These observations suggest that a genetic program exists prior to fusion which is refractory to subtle differences in the environment. However, environmental conditions exist that will suppress this program and prevent the co-expression of vastly different MyHC isoforms. Whether the absence of the L6 phenotype is due to the transition of the L6 nuclei, or to their complete down-regulation is unclear.

Since innervation has been suggested to be a key determining role in muscle fibre type (Jolesz and Sreter, 1981; Pette and Vrbova, 1985), the fate of L6 nuclei within both fast and slow muscle fibres was examined. Characterization of these fibres with a Mab specific for embryonic MyHC revealed accumulations of this isoform in small pockets around individual nuclei. These accumulations are small ($\leq$ 60 µm) and usually located at the periphery of the fibre, although in some instances they covered the entire cross-sectional area of the fibre. It is possible that these pockets of expression may represent localized areas of regeneration since developmental isoforms are re-expressed upon regeneration of muscle. However, this is unlikely since these accumulations persist through out the length of the study in histologically mature fibres. The re-expression of embryonic MyHC in the contralateral limb lasts approximately 14 days which is in agreement with other

published reports (D'Albis *et al.* 1988; D'Albis *et al.* 1989).

Such fusion events have been shown to occur between satellite cells and undamaged areas of muscle fibres (Robertson *et al.* 1992). However, this is still unlikely to account for accumulations of embryonic MyHC 56 days after injection, since proliferation of cells after transplantation peak at seven days and is finished by ten days after injection (Roberts *et al.* 1983)

The most likely explanation of these results is that these accumulations represent nuclear domains in which the L6 phenotype is being maintained. The fact that embryonic MyHC down-regulation is not correlated to innervation has been previously observed by other labs and confirms observations in homotypic myotubes that become innervated. The compartmentalization of MyHC observed in heterotypic fibres is similar to the subsynaptic localization of novel MyHCs induced by ectopic innervation of muscles by foreign neurons (Salviati *et al.* 1986). Although nuclear domains of embryonic MyHC expression are not observed in all heterotypic fibres examined, it is possible that these domains are undergoing a transition similar to the homotypic L6 myotubes discussed in Chapter 6. This transition would involve the up-regulation of IIX MyHC which should be observed in the majority of cases by four weeks after injection. The examination of large areas of heterotypic fibres in both the gastrocnemius and superficial tibialis anterior confirm this transition, as the great majority of fibres express IIX MyHC alone, or in conjunction with other fast isoforms. In both muscles there are large concentrations of IIX fibres which is remarkably different from the surrounding area.

It might be suggested that these fibres actually represent homotypic L6 fibres which have replaced the host muscle in this area, similar to myoblast transplantations in which muscles were extensively damaged, or completely destroyed. However, the observation that the X-gal staining does not extend through the entire length of the muscle suggests that these fibres are indeed the result of fusion events between host and donor myoblasts. A more likely explanation is that the section of the fibre that was analyzed contained nuclei that were predominantly donor-derived. Similar

analysis of the same fibre in an area several hundred microns away may reveal a completely different pattern of expression. This reasoning would also explain the absence of IIX labelling in several heterotypic fibres since, at this level, the nuclei within the fibre may be predominantly host-derived.

Interestingly, the appearance of such domains were only observed in fast muscles. Heterotypic fibres that were recognized by slow MyHC-specific Mabs showed only transient expression of the IIX phenotype, suggesting that the type of innervation may override ıe intrinsic L6 program of expression. This indicates that the maintenance of the IIX and embryonic MyHCs is due to a permissive environment found in the fast populations. The colocalization of fast mRNA transcripts can be found in normal adult rat hindlimbs (DeNardi *et al.* 1993; Campione *et al.* 1993). However, co-expression of the fast IIX and slow isoforms is not observed, suggesting that some type of restriction is placed upon the fibres. Alternatively, the expression of slow MyHC may be within the adaptive range of L6 myoblasts. An adaptive range represents all of the phenotypic potentials of a given myoblast population, and allows for some modulation of the final fibre type (Westgaard and Lomo, 1988). Analysis of the L6 MyHC expressional pattern has revealed the presence of low levels of embryonic slow MyHC protein (Chapter 5). Characterization of the MyHC mRNA profiles supported the presence of slow MyHC transcripts (Muthuchamy *et al.* 1992). Therefore, it is possible that this developmental potential of the L6 cells, which does not predominate in culture, could be replacing the more prevalent IIX phenotype under appropriate conditions (ie. innervation by a slow motoneuron). Since neither IIA or IIB MyHC proteins were observed *in vitro*, the L6 cells may be restricted from expressing those isoforms even when placed in the appropriate environment.

The retention of embryonic and/or IIX MyHC expression by donor nuclei incorporated into a vast excess of host sarcoplasm is direct evidence that individual nuclei within a muscle fibre can behave independently, and is in agreement with previous studies by others who examined the selective expression of acetylcholine

receptor subunits (Simon *et al.* 1992) and acetylcholine receptor-LacZ transgenes (Sanes *et al.* 1991) by subsynaptic nuclei. Nuclear domains for MyHC have also been shown to occur *in vitro* within human/mouse heterokaryons (Pavlath *et al.* 1989). While there is strong evidence from denervation/reinnervation experiments for the ability of motoneurons to transform the fibre types of adult muscles (Eldridge *et al.* 1984), there is also good evidence that the development of fibre types can occur normally in surgically (Butler and Cosmos, 1981) or chemically (Condon *et al.* 1990; Fredette and Landmesser, 1991) denervated embryos. These results support the theory that different myoblast populations have different inherent developmental potentials.

The results described here concur with recent findings from Stockdale's laboratory which follow the fate of cloned quail satellite cells (Feldman and Stockdale, 1992) and fetal myoblasts (DiMario *et al.* 1993) following injection into embryonic chick hindlimb buds. Their findings suggest the presence of genetically programmed fibre-type potentials in primary cultures of different myogenic lineages, albeit for much shorter periods after injection (DiMario *et al.* 1993). However, Stockdale's group details only the expression of homotypic fibres *in vivo*. Studies using MyHC expression as a marker in heterotypic fibres has produced results that are somewhat different from our findings here. Injection of either the mouse C2C12 cell line or cloned satellite cells into adult hindlimb muscles (Hughes and Blau, 1992) suggests that myoblasts adapt and change based on their environment. However, It could be that the donor cell populations that they injected, which express a vast array of MyHC isoforms *in vitro*, are much more plastic than the L6 cell line, and therefore, have an adaptive range that covers all possible muscle environmants.

In conclusion, the results of this study suggest that the endogenous "default" program of the L6 myoblasts which is expressed *in vitro* is also exhibited in fast environments *in vivo*. Although environmental influences may be overriding this program in slow environments, the appearance of slow MyHC in L6 myotubes *in vitro* suggests that these cells are exhibiting an adaptive range. This range would

consist of two different developmental potentials for the L6 myoblast population, and the final modulation is the result of environmental factors.

CHAPTER 8 - FINAL CONCLUSIONS AND SUMMARY

The purpose of this study was to determine if myoblasts obtained from early and late stages of development represent distinct myogenic lineages based on their developmental potential. To do this, the fate of myoblasts derived from embryonic day (ED) 14 (embryonic) and ED 20 (fetal) were analyzed *in vitro* to establish their default patterns of MyHC expression. To determine if the differences in these default programs represented the basis of a myoblast lineage, the cell populations were challenged by several *in vivo* environments. In addition, the developmental potential of a rat myoblast cell line (L6) was also examined both *in vitro* and after injection into regenerating muscles. The results presented here indicate that embryonic and fetal myoblasts exhibit the potential to form slow and fast muscle fibres, respectively. However, their full developmental potentials, which are not fully realized in culture, can only be observed in the appropriate environment such as the caudate-putamen. L6 myoblasts also exhibit a restricted potential being able to express either fast IIX or slow isoforms *in vitro* and *in vivo*. Therefore, these studies support the existence of myogenic lineages which can be modulated by environmental cues such as innervation. The intrinsic program established, which represents the lineage's adaptive range, determines the extent that the environment can modulate the final phenotype of the myoblasts. Furthermore, the limitations imposed by the intrinsic program occurs early in development, possibly before the expression of mrfs, and represent an event in myogenesis termed "delineation". This event follows myogenic determination and precedes myogenic differentiation.

To account for the different phenotypes of individual muscle fibres in adult mammalian muscle, several models have been presented which deal with differences in myoblast origin, innervation, mrf expression, or other environmental cues (Hughes *et al.* 1993; Stockdale, 1992; Donoghue and Sanes, 1994). The most basic models argue for a complete control by either the state of innervation or the genetic makeup of the myoblasts (see Figure 1.1). The former model suggests that all muscle fibres

are derived from a common pool of precursor myoblasts. The differences in muscle fibre type are then due to differences in the type of innervation received by the fibre. This model would suggest that all myoblasts are equal in their developmental potential and, therefore, that each population should react to a given environment in a similar fashion. However, upon culturing cells derived from ED 14 (embryonic) and ED 20 (fetal) in similar culture conditions, vastly different developmental potentials are observed (Chapter 2). Embryonic cell cultures revealed the presence of mononucleated slow type myocytes, while fetal cultures revealed the presence of large multinucleated myotubes expressing a fast phenotype. The observed phenotypes mirror the expressional pattern of these myoblast populations during development (Condon *et al.* 1990), and suggest that embryonic myoblasts become primary myotubes and fetal myoblasts become secondary myotubes. Similar results have also been observed in other species (Smith and Miller, 1992; Vivarelli *et al.* 1988; Stockdale and Miller, 1987). Although it could be argued that the different cell populations create their own microenvironment, the simplest explanation of these results is that different default patterns of expression exist within these populations. These results are corroborated by the observation that different classes of myotubes that represent all of the adult muscle phenotypes, exist after injection of the cell populations into the brain. The restriction of MyHC co-expression in these classes of myotubes is established prior to innervation and is similar to that observed in adult mammalian muscle (Jiao *et al.* 1993), indicating that the restrictions are due to intrinsic factors. Finally, the observations that homotypic myotubes consisting predominantly of L6 nuclei exhibit the same phenotype regardless of the muscle environment, indicates that this myoblast populations contains an intrinsic program that governs its final phenotype (Chapter 6).

All of the above observations support a model that suggests separate myogenic lineages exist for each muscle fibre type. In other words, each muscle fibre is made up of myoblasts derived from a single lineage, and these populations are restricted to one particular phenotype, regardless of the environment (see Figure 1.1).

However, the appearance of several MyHC isoforms in a single myotube after injection into the brain argues against such a model. Alternatively, innervation may be important for the down-regulation of some MyHC isoforms. Stronger evidence against such a model comes from the analysis of heterotypic fibres found in the hindlimb after fusion of L6 donor myoblasts to host myoblasts (Chapter 7). This model would suggest that the L6 cells would maintain their expression of IIX MyHC regardless of the environment. This is not the case since the normal L6 expression of fast IIX MyHC is not observed in slow fibres. This suggests that, under some circumstances, the expression of L6 nuclei is being modulated. While it could be argued that the L6 nuclei are being completely turned off, the presence of slow MyHC expression within a clonal population of L6 cells *in vitro* suggests that these cells may have the potential to become phenotypically slow.

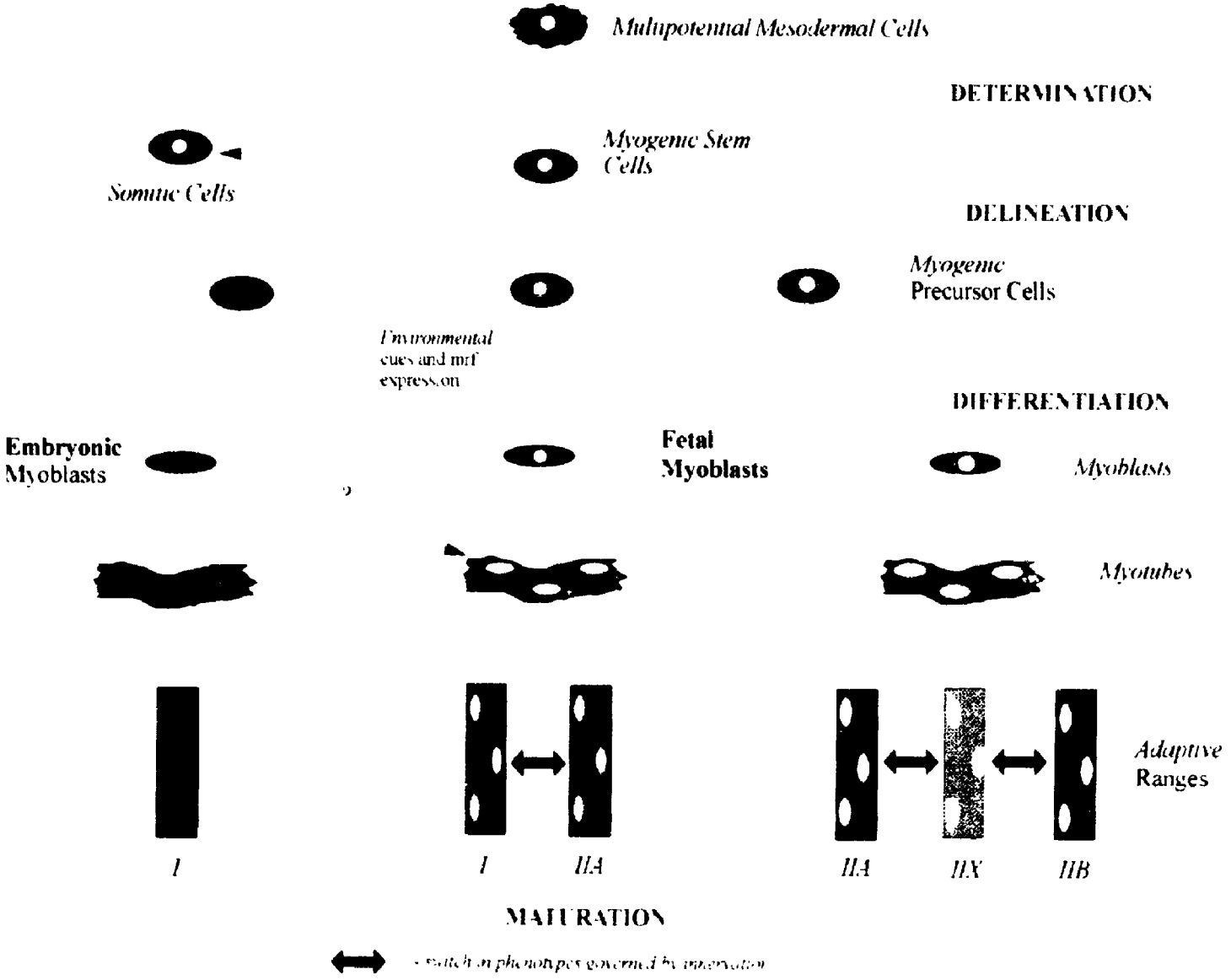
The results presented here are most consistent with a model in which both intrinsic and extrinsic factors play a role in the development of muscle fibre type. Models that have suggested a dual control on muscle phenotype have previously been suggested by several researchers. Stockdale and Miller (1992) have suggested the existence of three myogenic lineages in avian myogenesis. These lineages include two restricted populations - the slow and fast lineages - as well as an unrestricted population, the fast/slow lineage. The populations are separated temporally with the slow lineage only existing at early time points in development. Another model has been suggested by Hoh and colleagues (Hoh *et al.* 1989), which describes differences between myoblast populations derived from hindlimb muscles and jaw muscles. The restricted expression of a superfast MyHC to fibres derived from the latter class regardless of the environment, has led to the proposal that myoblasts exist as different allotypes. A myogenic allotrope consists of a myoblast population that has a limited array of phenotypic expressions. This developmental potential may overlap with another such allotrope, however, certain potentials would be restricted to only one of the allotropes. A similar model, involving adaptive ranges, has been described by Westgaard and Lomo (1988). This model suggests that each myoblast population

has a specific range of developmental potentials, and that the final fibre type is dictated by environmental influences. However, this potential is restricted to the adaptive range inherent in the population.

The observations described in this study suggest the existence of dual controls of muscle fibre type as illustrated by the model presented in Figure 8.1. To properly present a model, it is necessary to place the establishment of myogenic lineages and the level(s) of environmental control in the context of muscle development. During normal myogenesis, all myoblasts are derived from a common myogenic stem cell population which resides in the somite and is derived from a multipotential mesodermal cell population (Buffinger and Stockdale, 1994; Rong *et al.* 1992). Further development of these stem cells results in the establishment of myogenic precursor cells, which become restricted into different developmental pathways or lineages. Some of the myogenic precursors remain within the somite to form the axial muscles (ie. somitic lineage), while others migrate out of the somite into the developing limbs (Ordahl and LeDouarin, 1992). These migrating myogenic precursor cells may correspond to myogenic lineages which migrate into the limbs at different stages of development to give rise to primary and secondary myotubes (Seed and Hauschka, 1984). While these cells are determined to become myoblasts, they do not express early myogenic markers or mrfs at this time (Tajbakhsh and Buckingham, 1994). Environmental factors may act upon these cells to cause them to develop into myoblasts which then fuse under the appropriate conditions to form myotubes with different developmental potentials.

In this proposed model, different myogenic lineages are established which each exhibit an array of developmental potentials (ie. an adaptive range). This potential may only be partially realized *in vitro*, but can be fully observed in an appropriate *in vivo* environment, such as the caudate-putamen. When such a lineage is placed within a muscle environment and allowed to fuse with innervated fibres, environmental cues may limit the phenotypic expression to a single fibre type. However, the final phenotype of the myoblast is restricted to the adaptive range in-

Figure 8.1 Determination of muscle fibre type based on the establishment of myogenic lineages which have adaptive ranges of phenotypic expression. A single population of multipotential mesodermal cells develops into myogenic stem cells. The stem cells undergo delineation, giving rise to either somitic myoblasts or one of three developmental myogenic lineages - one embryonic and two fetal lineages. Differentiation of these lineages occurs in the presence of appropriate environmental cues and the expression of myogenic regulatory factors (mrfs), and results in myotubes which exhibit characteristic phenotypic potentials, otherwise known as adaptive ranges. Modulation within these adaptive ranges occurs upon innervation resulting in the expression of a single MyHC isoform. Specifically, the embryonic myogenic lineage has a limited phenotypic potential, giving rise to slow only fibres. However, they may also contribute to one of the fetal myogenic lineages - slow/fast IIA. The two fetal lineages give rise to slow/fast and fast only muscle fibres.


Multipotential Mesodermal Cells
DETERMINATION
Myogenic Stem Cells
Somitic Cells
DELINEATION
Myogenic Precursor Cells
Environmental cues and mrf expression
DIFFERENTIATION
Embryonic Myoblasts
Fetal Myoblasts
Myoblasts
Myotubes
Adaptive Ranges
I
I
IIA
IIA
IIX
IIB
MATURATION
switch in phenotypes governed by innervation

herent to the lineage. In his model, the establishment of the myogenic lineages predates the final innervation of the muscle fibres since embryonic and fetal cell populations express different phenotypes *in vitro* and within the caudate-putamen prior to innervation. It is also likely that the establishment of myogenic lineages precedes fusion between the myoblasts. This is indicated by the observation that muscle heterokaryons derived from myoblasts of fetal and embryonic origin exhibit short term maintenance of their characteristic MyHC profile. Also, L6 myoblasts fusing onto fast IIA and IIB fibres in regenerating muscle retain their expression of IIX MyHC. The existence of myogenic lineages existing prior to fusion is also inherent in the models described by others.

Further observations indicate that the establishment of particular myogenic lineages may occur prior to the expression of early myogenic proteins (such as desmin) and possibly even before mrf expression. The existence of myogenic regulatory factors which do not express mrfs has been recently documented by Tajbakhsh and Buckingham (#2603). The appearance of several adult fast populations after injection of ED 14 - derived cells into the caudate-putamen suggests that the embryonic cell population contains myogenic lineages that differentiate in culture (the slow only population of fibres) as well as myogenic precursor cells that do not differentiate *in vitro*. The "extra" myogenic lineages are observed only in the fetal cultures since at earlier stages they represent myogenic precursor cells, similar to satellite cells of mature muscle, while at later time points, they have developed into myoblasts. These cells only reach the next stage of differentiation in the presence of specific environmental cues which are absent in culture, but are present in the caudate-putamen. Such cues may be necessary for the up-regulation of mrfs which promote the transition of myogenic precursor cells into myoblasts. Recently, it has been proposed that of such factors are present in the neural tube, since removal of the neural tube in quail has led to an absence of muscle (Stern and Hauschka, 1995; Buffinger and Stockdale, 1994). Complete differentiation of the myogenic lineages in the caudate-putamen supports the

existence of such a factor(s). Since similar classes of myoblasts are observed upon injection of embryonic cells (when the later myogenic lineages exist as muscle precursor cells) or fetal cells (when these same lineages exist as myoblasts), this indicates that the lineages are established prior to or in conjunction with the establishment of myogenic precursor populations. Therefore, it appears that the establishment of myogenic lineages is an early stage of myogenic differentiation. This stage may be termed myogenic delineation and precedes the establishment of the mature myoblast phenotype.

LIST OF ORIGINAL CONTRIBUTIONS

1. Myoblasts obtained from embryonic day 14 (ED 14) and ED 20 show different developmental potentials of MyHC expression *in vitro*. ED 14 (embryonic) myoblasts exhibit a slow specific phenotype, while ED 20 (fetal) myoblasts exhibit a neonatal/adult fast phenotype. This pattern of expression is similar to the reported pattern of MyHC expression of primary and secondary myotubes during development.

2. Embryonic and fetal myoblasts will fuse with each other in culture to form myotube heterokaryons, indicating that no physical barriers exist which prevent these two populations from fusing with each other.

3. Individual embryonic or fetal - derived nuclei maintain their specific developmental potential within these myotube heterokaryons. This suggests that the default pattern of MyHC expression is established prior to fusion of myoblasts to form myotubes.

4. Injection of cultured cells obtained from the hind limb bud and adjacent spinal cord of ED 14 rat fetuses into the caudate-putamen results in the formation of an osteogenic core surrounded by well developed muscle fascicles. This indicates that the factors necessary for morphogenesis of the hind limb are present within the injured caudate-putamen.

5. Embryonic cell injection sites contain three general classes of myotubes; a fast population, a fast/slow population and a slow population. Fetal cell injection sites contain only fast and fast/slow myotube classes. This suggests that a specific class of myoblasts with the potential to form slow muscle fibres only exists at early time points in gestation.

6. Myotubes which belong to the fast/slow class of myotubes specifically express fast IIA MyHC only. Co-localization of slow MyHC with either fast IIX or fast IIB does not occur indicating that there is a restriction in the co-expression of MyHC isoforms.

7. All MyHC isoforms can be observed in myotubes expressing NCAM along their entire surface. This indicates that innervation is not essential for the initial appearance of MyHCs. In addition, all of the different classes of myotubes could be observed prior to innervation suggesting that the restrictions in MyHC co-expression are intrinsically based.

8. L6 myoblasts express two other MyHC isoforms in addition to embryonic MyHC. These isoforms are the slow embryonic and adult fast IIX isoforms.

9. Homotypic fibres of L6 myoblasts *in vivo* maintain their in vitro developmental potential, expressing both embryonic and fast IIX MyHCs, regardless of the muscle environment that they are within.

10. Although L6 homotypic fibres undergo a MyHC transition from embryonic to fast IIX *in vivo*, neither the disappearance of the embryonic MyHC, nor the appearance of the IIX MyHC is correlated to innervation of these fibres.

11. L6 myoblasts maintain their developmental potential upon fusing with donor muscle fibres that are phenotypically fast IIA or IIB.

12. L6 myoblasts do not maintain their expression of embryonic or fast IIX MyHC expression in heterotypic slow fibres.

13. Myogenic lineages exist in which intrinsic programs establish characteristic developmental potentials early in myogenesis. The intrinsic program established restricts an individual muscle fibre in its ability to form different fibre types. The restriction consists of an adaptive range which allows for some modulation of phenotype by external factors.

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